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Chemotherapy-induced Testicular Toxicity



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Submitted for PhD Thesis examination at the University of Edinburgh 2020

Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and work of all others is duly acknowledged in the text. I also acknowledge all assistance given to me during the course of these studies.

The majority of the work presented in Chapter 1 was previously published in Reproduction as a review paper 'How does chemotherapy treatment damage the prepubertal testis?' by **C.Allen**, secondary supervisors of PhD studies Dr F.Lopes and Prof R.Mitchell, and principal supervisor Prof N.Spears. This review was drafted primarily by myself.

The work presented in Chapter 3 was previously published in Molecular Human Reproduction as an original research paper 'Comparative gonadotoxicity of cisplatin and carboplatin on prepubertal mouse gonads' by **C.Allen**, secondary supervisors of PhD studies Dr F.Lopes and Prof R.Mitchell, and principal supervisor Prof N.Spears. I participated in the experimental design of the study, led experiments, analysed data, prepared figures and wrote the manuscript.



Caroline Allen

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Abstract

Childhood cancer survival rates have continued to increase in recent decades, with current survival rates of around 80%. This is due in large part to improvements in cancer treatment including the use of chemotherapy drugs. Chemotherapy treatment during childhood can adversely affect the reproductive system, resulting in infertility in adulthood. Gonadotoxicity and associated infertility risk of chemotherapy agents used to treat childhood cancer is, however, debated and often based on adult patients. Clinically, there are no fertility preservation options available for prepubertal boys since, unlike adult men, they do not yet produce mature spermatozoa for routine sperm cryopreservation. Alternatively, cytoprotective agents could be given alongside the cancer treatment to mitigate chemotherapy-induced damage to healthy reproductive tissues, without interfering with the ability to treat the cancer. In order to develop cytoprotective agents, a thorough understanding of the underlying mechanism of action and molecular targets of chemotherapy agents are required, specifically in relation to the prepubertal testis. In this thesis, two models of the prepubertal testis were used to study chemotherapy-induced testicular toxicity, a mouse prepubertal testis tissue culture model and a rat GC-6spg cell line model, representative of spermatogonial stem cells.

Firstly, the gonadotoxicity of first- and second-generation drugs of the platinum alkylating-like drug class were compared, to determine whether the newer generation drug carboplatin was less gonadotoxic than cisplatin, as reported for other organ specific toxicities. This proved not to be the case, with carboplatin found to be equally gonadotoxic to the prepubertal testis as cisplatin, reducing the density of proliferating germ cells. Given these results, there is no indication that modifying treatment regimens to include carboplatin instead of cisplatin would spare the testis from chemotherapy-induced testicular toxicity.

The role of reactive oxygen species (ROS) and oxidative stress in chemotherapy-induced damage, as well as the protective effects of antioxidants, against cisplatin-,

doxorubicin- or vincristine-induced gonadotoxicity were also examined. Cisplatin and doxorubicin are known to be gonadotoxic, with the general underlying mechanisms of action linked to oxidative stress. In contrast, vincristine has not been considered to be gonadotoxic based on adult fertility rates, and has also not been thought to induce oxidative stress as a mechanism of action. The results from both models, however, indicate that vincristine is gonadotoxic to the prepubertal testis, causing accumulation of germ cells into the centre of seminiferous tubules in the prepubertal testis model. Preliminary data could not rule out a role for ROS and oxidative stress in chemotherapy-induced damage: further work is required to optimize the techniques for assessment of ROS and oxidative stress. Co-treatment with antioxidants melatonin, n-acetylcysteine (NAC) and lycopene produced differing results depending on the chemotherapy drug. High concentrations of melatonin and NAC partially protected against cisplatin-induced damage, whereas NAC enhanced vincristine cytotoxicity and lycopene treatment alone was cytotoxic to the GC-6spg cell line.

Overall, these results are a step towards furthering our understanding of the underlying mechanism of toxicity of commonly used chemotherapy agents in the prepubertal testis. Drugs that are currently considered less gonadotoxic, often from limited clinical information, should be investigated for their direct effects on the prepubertal testis since this may differ from the adult testis. In addition, antioxidants may be beneficial or detrimental in protecting the testis from chemotherapy-induced testicular toxicity.

Lay Summary

Around 80% of children who are diagnosed with cancer are surviving into adulthood. This vast improvement in survival rates is due to advances in cancer treatment, partly through increased use of chemotherapy drugs, toxic agents that kill off the cancer cells. These drugs can also damage healthy tissues, leading to life-long health complications. For children, the chemotherapy treatments can damage the reproductive tissues and lead to infertility in adulthood. The reproductive tissues in males produce sperm, needed to father children. Currently, doctors can protect the fertility of older males by freezing sperm samples that could then be used later to father children. Young boys, however, may not yet be able to produce any sperm if they have not yet undergone puberty, and where that is the case, doctors cannot protect the fertility of these young boys. Scientists are therefore aiming to develop a system to protect the testes of these young boys from the chemotherapy drugs, whilst they kill off the cancer.

In this thesis, the method behind the toxic damage caused by chemotherapy drugs to the testes of young boys are examined. With more information on how these drugs work, a way to protect the testes from damage could be developed and tested. Since it is hard to study this in humans, instead animal models are used in this thesis, including testis tissue taken from young male mice and a line of cells from rats, which are derived from immature sperm cells. These tissues/cells are grown in a dish and exposed to different chemotherapy drugs that can be given to children to treat the cancer.

First, closely related chemotherapy drugs that kill off the cancer in the same way were compared to each other, to determine whether the newer version of the drug was less toxic than the older one: if so, this newer drug could be used instead to limit the amount of damage to the reproductive tissues. This, however, was not the case for the drugs examined, and the newer drug tested was just as toxic as the earlier version of that drug. Secondly, chemotherapy drugs that are commonly used to treat

childhood cancers were further investigated to determine the method behind the toxicity. The results are preliminary, but show unexpectedly that a drug not known to cause infertility is damaging to the immature reproductive tissues and sperm cells. Thirdly, potential protective agents were tested to see whether they could limit the damage caused by the chemotherapy drugs. These agents were able to partially protect the immature reproductive tissues or cells from the chemotherapy-induced damage, whereas others enhanced the damage caused by the chemotherapy drugs.

Overall, the results from this thesis are a step towards understanding how toxic different chemotherapy drugs are and how they cause damage to the testes, as well as discovering a compound that will protect the testes from the damage.

Publication arising from this work

Allen CM, Lopes F, Mitchell RT, Spears N. How does chemotherapy treatment damage the prepubertal testis? *Reproduction*, 2018, 156(6): R209-233.

Adapted and is the basis of literature review in Chapter 1 and attached in Appendix A. This work is licensed under a Creative Commons Attribution 4.0 International License

Allen CM, Lopes F, Mitchell RT, Spears N. Comparative gonadotoxicity of cisplatin and carboplatin on prepubertal mouse gonads. *Molecular Human Reproduction*, 2020, 26(3): pp.129-140.

Chapter 3, paper included. This work is licensed under a Creative Commons Attribution 4.0 International License

Tharmalingam MD, Matilionyte G, Wallace WHB, Stukenborg JB, Jahnukainen K, Oliver E, Goriely A, Lane S, Guo J, Cairns B, Jorgensen A, **Allen CM,** Lopes F, Anderson RA, Spears N and Mitchell RT 'Cisplatin and carboplatin result in similar gonadotoxicity in immature human testis with implications for fertility preservation in childhood cancer'; revised version of manuscript currently under review by *BMC Medicine*.

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Abstracts for poster presentations

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Comparison of toxicity of chemotherapy drugs cisplatin and carboplatin on prepubertal mouse gonads

Written and presented at Fertility 2018, ISFP 2019 and SDBG 2018 (no abstract submitted)

Abstract for oral presentations

Caroline Allen, Federica Lopes, Rod Mitchell, Norah Spears

Comparison of toxicity of chemotherapy drugs cisplatin and carboplatin on prepubertal mouse gonads

Written and presented at European Testis Workshop 2018 and SDBG 2019 (no abstract submitted)

Caroline Allen, Aisha Parveen, Federica Lopes, Rod Mitchell, Norah Spears

The impact of vincristine treatment on prepubertal mouse testis

Abstract written by Caroline Allen and presented at Fertility 2020 by Norah Spears

Caroline Allen, Federica Lopes, Rod Mitchell, Norah Spears

Can antioxidant treatment protect a rat spermatogonial stem cell line against chemotherapy-induced damage?

Written and will be presented at postponed ETW 2020/21 conference

Assistance given during this PhD

All of the work detailed within this thesis was performed by myself, with the following exceptions:

Chapter 2: Emma Guy (masters student) under my supervision performed the immunohistochemistry and analysis of age-matched controls MVH+/Ki67+.

Chapter 4: Aisha Parveen (honours student) under my supervision performed sectioning, immunohistochemistry and analysis of the dose-response data for vincristine clinical preparation ('n'=4) and mannitol cultures. She also prepared Figure 4.1B. Catherine Stewart (honours student) under my supervision performed sectioning, immunohistochemistry and analysis of vincristine short- and long-term culture experiments.

Chapter 5: Eva Baxevanidi (student) performed the sectioning and immunohistochemistry of doxorubicin powder cultures. Emma Guy performed the sectioning and immunohistochemistry of time-course cultures and performed the analysis of oxidative stress marker 8-OHdG+.

Histology: Vivian Allison or Louise Dunn conducted processing of mouse testis tissue after fixation and embedding into paraffin wax. Louise Dunn fixed the back skin tissue that was collected by Atanaska Velichkova.

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Abbreviations

$^1O_2$; singlet oxygen

8-OHdG; 8-hydroxy-2'-deoxyguanosine

ABP; androgen binding protein

Abs; absorbance

ABVD; doxorubicin, bleomycin, vinblastine and dacarbazine

ALC; adult Leydig cells

AMH; anti-Müllerian hormone

ART; assisted reproductive technologies

ATM; ataxia-telangiectasia mutated

ATR; ATM- and Rad3-related

B[a]P; benzo[a]pyrene

bFGF; basic fibroblast growth factor

BM-MSCs; bone marrow mesenchymal stem cells

BMP; bone morphogenetic protein

BSA; bovine serum albumin

BTB; blood testis barrier

CAT; catalase

CC; cleaved caspase

CED; higher cyclophosamide equivalent doses

CHOP; cyclophosphamide, doxorubicin, vincristine and prednisolone

CSF1; macrophage colony stimulating factor

CVP; cyclophosphamide, vincristine and prednisolone

CYC; cyclophosphamide

DNA-PKs; DNA-dependent protein kinase

dpc; days post coitum

E; embryonic day

EC50; half maximal effective concentration

EGF; epidermal growth factor

FLC; fetal Leydig cells

FSH; follicle stimulating hormone

GC; germ cells

GCSF; granulocyte colony-stimulating factor

GDNF; glial cell-derived neutrophic factor

GDP; guanosine diphosphate

GIM-I; ginseng intestinal metabolite I

GPx; glutathione peroxidases

GR; glutathione reductase

GSH; reduced glutathione

GSP; glutathione transferase

GSSG; oxidized glutathione

GST; glutathione-S-transferase

GSX; glutathione-X-conjugate

GTP; guanosine-5'-triphosphate

GTPx; glutathione peroxidase

GW; gestational week

H_2O_2; hydrogen peroxide

hCG: human chorionic gonadotrophin

HNE; 4-hyroxylnonenal

HPG; hypothalamic-pituitary-gonadal

hr: hour

hrs: hours

ICSI; intracytoplasmic sperm injection

INSL3; insulin-like peptide 3 hormone

IVF; in vitro fertilization

KSR; knockout serum replacement

L-15; leibovitz media

LC; Leydig cells

LH; luteinizing hormone

LIF; leukaemia inhibitory factor

MAPs; microtubule-associated protein

MDA; malondialdehyde

MEM; minimum essential media

MDR; multi-drug resistance

MOE; *Moringa oleifera* plant

MOPP; nitrogen mustard, vincristine, procarbazine and prednisolone

MTCO; microtubule organising centre

mths; months

NAC; N-acetylcysteine

NADPH; nicotine adenine dinucleotide phosphate

$NO^{\bullet}$; nitric oxide

$O_2^{\bullet -}$; superoxide radicals

$OH^{\bullet}$; hydroxide radical

$ONOO^-$; peroxynitrite

PBS; phosphate buffered saline

PDGF; platelet-derived growth factor

PGCs; primordial germ cells

PMCs; peritubular myoid cells

pnd; postnatal day

POC; perfusion open and closed

PRX; peroxiredoxin

RNS; reactive nitrogen species

ROS; reactive oxygen species

SC; Sertoli cells

SCF; kit ligand stem cell factor

SCO; Sertoli cell only tubules

SDF-1/CXCL12; stromal derived cell factor

SSC; spermatogonial stem cell

SRY; sex determining region Y

SOD; superoxide dismutase

SOX9; SRY-box 9

TBARs; thiobarbituric acid reactive substances

TFI; tubular fertility index

TRX; thioredoxin

yrs; years

wks; wks

Chapter 1. General Introduction

The majority of this Chapter has been previously published as a literature review (Allen *et al.*, 2018): this review was drafted primarily by myself (see Appendix A for publication). A number of topics have been expanded upon and the content of the review updated as appropriate.

1.1 Introduction

Overall, the childhood cancer survival rate has increased substantially in recent decades, with the current 5-year survival rate at around 80%, compared to about 58% in the late 1970s (Miller *et al.*, 2016). This marked advance, due in large part to improvements in cancer detection and treatment, including the use of chemotherapy drugs, has led to a growing population of long-term childhood cancer survivors. Chemotherapy drugs, however, do not exclusively target malignant cells but also elicit side effects due to off-target damage to healthy tissues (reviewed in Robison and Hudson, 2014). Given this situation, research is increasingly focusing on preventing or diminishing chemotherapy-induced damage to otherwise healthy organs, to improve the quality of life for childhood cancer survivors (Gibson *et al.*, 2018). For younger patients, detrimental effects of treatment on fertility can be of a major concern (Zebrack *et al.*, 2004; Ellis *et al.*, 2016). This is particularly problematic for male survivors of childhood cancer, given the current lack of available fertility preservation treatments. These young prepubertal boys, unlike adult patients, do not yet produce mature spermatozoa that can be used for routine sperm cryopreservation (reviewed in Wyns *et al.*, 2010; Wallace, 2011; Tournaye *et al.*, 2014; Mitchell *et al.*, 2017. Research in animal models has been successful in showing the potential of fertility preservation techniques, which could hypothetically be developed for clinical use (reviewed in Picton *et al.*, 2015; Giudice *et al.*, 2017; Goossens *et al.*, 2020).

A potential fertility preservation strategy could involve the development of interventions administered before and/or during chemotherapy treatment to prevent the damage from occurring in the first instance, thus protecting the fertility potential of the patient. Cytoprotective agents that specifically protect the prepubertal testis against chemotherapy-induced damage, without interfering with the toxicity to cancer cells, could be employed as part of the chemotherapy treatment regimen. However, research is required to further our understanding of the underlying mechanism of action and molecular targets of chemotherapy drugs on the prepubertal testis to identify such protective agents. At present, much of our knowledge has been derived from studies in adults; however, due to inherent differences in the testis when comparing the adult testis to the prepubertal, new research is needed to focus specifically on the prepubertal testis. This knowledge is essential to discover and develop cytoprotective agents to protect the prepubertal testis from chemotherapy-induced damage.

This PhD aims to investigate chemotherapy-induced damage to the prepubertal testes using a combination of a mouse testis culture system (Sato *et al.*, 2011a; Lopes *et al.*, 2016) and a cell line culture system, where the cell line is representative of spermatogonial stem cells (SSCs) derived from adult rats (van Pelt *et al.*, 2002). Different generations of drugs of the same class will be compared to determine whether newer generations of drugs are less gonadotoxic than the older as a fertility preservation strategy. The gonadotoxicity and underlying mechanism of action of commonly used chemotherapy agents in cancer treatment protocols for children will be determined. Finally, the ability of selected antioxidants to protect against chemotherapy-induced damage to the prepubertal testes/SSCs will be investigated.

1.2 Development of the Testis

The testis is responsible for producing mature spermatozoa and also for production of the main male reproductive hormone, testosterone. During prepuberty, the testis was originally thought to be relatively inactive, based on studies demonstrating few

morphological changes and a lack of hormone production during this period (reviewed in Shalet, 1980; Rey, 1999) . However, further detailed analysis has shown that the prepubertal testis undergoes important developmental processes which are required for normal adult functioning as shown in humans (reviewed in Chemes, 2001) and mouse models (Vergouwen *et al.*, 1993) (overview in Figure 1.1). This section will outline what is currently known regarding testis development, focusing primarily upon human development. Many studies, however, have relied upon animal models to observe developmental changes due to the challenges of studying the human testis; information about non-human species will be specified where relevant.

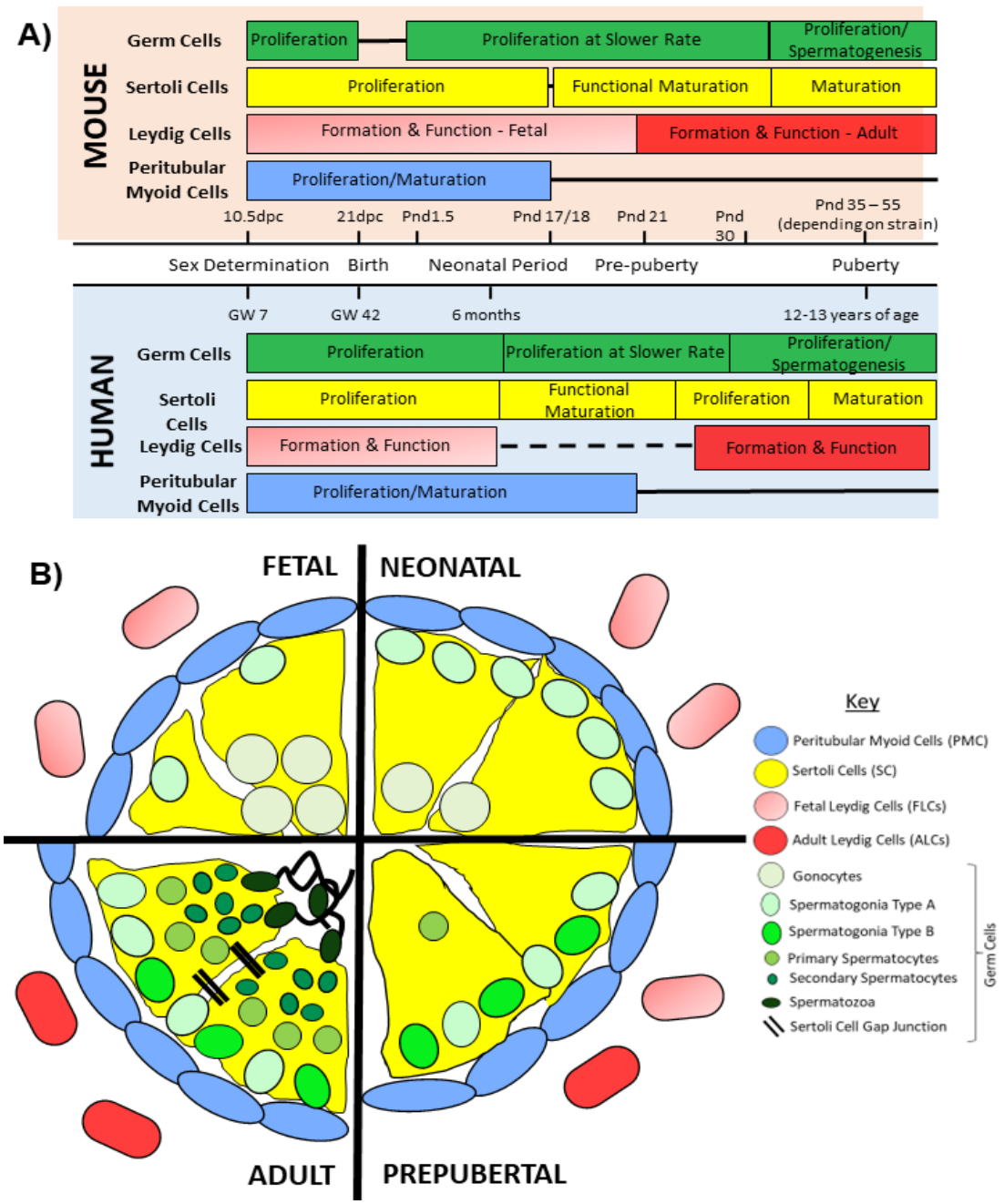


Figure 1.1: Developmental timeline of testis. A) Relative timeframe of important developmental processes taking place between fetal development and puberty in humans (reviewed in Chemes, 2001) and the mouse model (Vergouwen *et al.*, 1993). Solid line indicates no activity of the cells at the relative time points and dashed line represent the unknown nature of Leydig cell development during this timeframe B) Comparison of the histology of the testis throughout development in the human, from fetal development through to the adult testis. ALC; adult Leydig cells, dpc; days post coitum, FLC; fetal Leydig cells, GC; germ cells GW; gestational week, LC; Leydig cells, PMC; PMCs, pnd; postnatal days, SC; Sertoli cells. Created by myself and published in Allen *et al.* (2018).

1.2.1 Fetal Life

The testis forms during early fetal life from a bipotential gonadal ridge that forms from intermediate mesoderm and the underlying mesophonros. These cells give rise to the somatic cell population of the testis. The primordial germ cells (PGCs), which will eventually form mature spermatozoa in the adult, form early during development before or during gastrulation around weeks two/three post-conception in humans and embryonic day (E) 6-7 in mice (reviewed in De Felici, 2013; Svingen and Koopman, 2013; Stukenborg *et al.*, 2014; Mäkelä *et al.*, 2019). The PGCs are induced to form by the action of signalling molecules including bone morphogenetic protein (BMP) and WNT signalling molecules/pathways. In humans, PGCs are thought to form from the posterior epiblast or dorsal amnion cells, through the action of signalling factors produced by the epiblast, amnion or hypoblast (reviewed in Mäkelä *et al.*, 2019). Signalling factors including BMP4, leukaemia inhibitory factor (LIF), kit ligand stem cell factor (SCF) and epidermal growth factor (EGF) are thought to be involved in inducing formation of human PGCs from pluripotent stem cells (Sasaki *et al.*, 2015). In mice it is established that PGCs form from the posterior proximal epiblast by the action of signalling molecules such as BMP4, BMP8b produced by the surrounding extra-embryonic ectoderm and BMP2 by the visceral endoderm (Lawson *et al.*, 1999; Ying *et al.*, 2000; Ying and Zhao, 2001). By week three, the PGCs are located within the yolk sac endoderm and are incorporated into the hindgut/midgut during body folding. The PGCs migrate from their original position through the hindgut to populate the gonadal ridge by week five (E10 in mice) (Witschi, 1948 as cited Mäkelä *et al.,* 2019). This process of migration is achieved through passive displacement due to growth/embryo folding but also by active migration guided by signals produced by the gonadal ridge including factors such as the chemokine stromal derived cell factor (SDF1 or CXCL12) and SCF (reviewed in Barton *et al.*, 2016). In addition, it has been shown in humans that the PGCs migrate to the gonadal ridge along with autonomic nerve fibers (Møllgård *et al.*, 2010). During migration and upon arrival within the gonadal ridge, the PGCs proliferate (reviewed in De Felici, 2013). Within the gonadal ridge, the Sertoli cells differentiate from precursor cells under transient expression

of the *Sry* (sex determining region Y) gene, which drives production of the SOX9 (SRY-box 9) protein by week seven (E10.5/11 mice). The Sertoli cells engulf the migrated PGCs, which are now non-migratory and classified as gonocytes, resulting in the formation of seminiferous cords containing Sertoli cells with centrally located gonocytes (Hanley *et al.*, 2000). The Sertoli cells are key drivers in the differentiation and function of other cellular components of the testis, including the Leydig cells and peritubular myoid cells (PMCs) (Wen *et al.*, 2016) (Fig 1.1). The Sertoli cells also produce anti-Müllerian hormone (AMH) which acts upon the Müllerian ducts to bring about their regression and so prevent formation of female structures including the fallopian tubes, uterus and upper portion of the vagina (Aksglaede *et al.*, 2010). The hypothalamic-pituitary-gonadal (HPG) axis is active during fetal life, producing the gonadotrophins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are essential for the formation, maturation and function of key somatic cells including the Sertoli and Leydig cells, respectively (reviewed in Stukenborg *et al.*, 2018a)

During human fetal development, the gonocytes proliferate into early neonatal life, and differentiate into pre-spermatogonia during late fetal and early postnatal life (reviewed in Culty, 2009). This occurs asynchronously, with the gonocytes having greater proliferative activity than the pre-spermatogonia. In the rodent model, however, the proliferation of the gonocytes ceases during late fetal life and resumes during the early postnatal period (Ferrara *et al.*, 2006; Mitchell *et al.*, 2008; Wu *et al.*, 2009). Sertoli cells actively proliferate during fetal development under the control of multiple hormones, particularly FSH. This period of proliferation is very important in determining adult numbers of Sertoli cells (reviewed in Sharpe *et al.*, 2003). Between the seminiferous cords is the interstitial compartment where multiple different cells reside including the Leydig cells, PMCs, vascular endothelial cells and smooth muscle cells which all have a role in testis development and function (reviewed in Heinrich and DeFalco, 2019). The endocrine somatic Leydig cells are composed of different cell populations throughout an individual's lifespan. By week eight of gestation, the fetal

Leydig cells (FLCs) produce testosterone that stabilizes the Wollffian ducts, which later form the epididymis, ductus deferens and seminal vesicles (Del Valle *et al.*, 2017; reviewed in Mäkelä *et al.*, 2019). Testosterone production is key for masculinization of the male external genitalia which occurs during a specific window of masculinization during weeks 8-14 in humans (E15.5-19.5 in rodents) (Welsh *et al.*, 2008). Regulation of testosterone production during the MPW differs between humans and rodents, with paracrine regulation in rodents and human chorionic gonadotrophins (hCG) stimulation in humans (reviewed in Sharpe, 2020). During fetal development, androgen receptors are expressed in the testis specifically within PMCs and interstitial cells, but not in FLCs (Majdic *et al.*, 1995). The FLCs ultimately regress after birth and are replaced by adult Leydig cells (ALCs) during puberty in rodents (reviewed in Habert *et al.*, 2001; Chen *et al.*, 2009; Svechnikov *et al.*, 2010); however, the presence and/or function of the FLCs have recently been shown to be important in the formation and function of the ALCs (Su *et al.*, 2018). Whether FLCs and ALCs are distinct population of cells or share a common stem/progenitor cell lineage is at present unclear, however stem ALCs have been observed in the human and rodent fetal testis (Kilcoyne *et al.*, 2014; reviewed in Teerds and Huhtaniemi, 2015). These stem ALCs express androgen receptors, and androgen action during fetal development is essential for later programming of adult testosterone production (Kilcoyne *et al.*, 2014). Indeed, further studies have shown the importance of autocrine action of testosterone upon stem ALCs for subsequent Leydig cell maturation, steroidogenesis and maintenance in adulthood (O'Hara *et al.*, 2015). PMCs are thought to develop from interstitial cells where multiple layers of these cells, along with extracellular matrix proteins, surround and form the wall of the seminiferous cords: in mice, a single layer forms (Davidoff *et al.*, 1990). PMCs contribute to the basement membrane, providing structural support during testis development, and will function in the adult to aid movement of mature sperm towards the lumen (reviewed in Mayerhofer, 2013; Svingen and Koopman, 2013).

The testes descend during fetal life from their original developmental position in the abdomen into their final position in the scrotum. This occurs through two phases known as the trans-abdominal (10^{th} – 15^{th} weeks) and inguinoscrotal phases (25^{th} – 35^{th} week) (reviewed in Bay *et al.*, 2011; Hutson *et al.*, 2015). The trans-abdominal phase is dependent on production of insulin-like peptide 3 hormone (INSL3) produced by the Leydig cell population (Nef and Parada, 1999; Zimmermann *et al.*, 1999). INSL3 causes shortening and swelling of the gubernaculum (a mesenchymal structure connecting the testis to the inguinal area), which guides testis descent by anchoring the testis to the inguinal region as the embryo develops and lengthens (Kubota *et al.*, 2002). The inguinoscrotal phase, where the gubernaculum migrates into the scrotum bringing along the testis as it descends, is androgen dependent (Hutson, 1986). In mice, the trans-abdominal phase takes place during fetal life, whereas the inguinoscrotal phase occurs after birth by postnatal day (pnd) 10 (reviewed in Hutson *et al.*, 2015).

1.2.2 Neonatal Life

During the first three months of neonatal life, the reproductive system undergoes a short period of activity known as "mini-puberty", where the HPG axis is active (reviewed in Kuiri-Hänninen *et al.*, 2014). The origin of the Leydig cells that produce testosterone during "mini-puberty" is unknown, possibly forming from precursor cells or from the FLCs (reviewed in Svechnikov *et al.*, 2010). The function of "mini-puberty" has yet to be clarified, but has been postulated to be linked to later reproductive function in the adult (reviewed in Copeland and Chernausek, 2016). This short neonatal activity has also been observed in the primate animal model during neonatal development but does not appear to occur in rodents, where infancy overlaps with early stages of pubertal development (reviewed in Chemes, 2001; Kelnar, 2002).

The volume of the testis increases six-fold during "mini-puberty" due to FSH-stimulated Sertoli cell proliferation and protection from apoptosis; 93-95% of the

seminiferous tubule mass is attributed to the Sertoli cells at this stage (Müller and Skakkebaek, 1983; Rey *et al.*, 1993; reviewed in Petersen and Söder, 2006). This increase in cell number results in an overall increase in seminiferous cord length but not diameter, as each Sertoli cell maintains contact with the basement membrane, the cells spreading lengthwise (reviewed in Chemes, 2001). Therefore, in a cross sectional area of the seminiferous cord it may appear as though the cell density remains stable or even decreases during this period of development, which can be misleading (reviewed in Chemes, 2001). The resulting number of Sertoli cells will ultimately determine sperm production in the adult, with each Sertoli cell able to support a species-specific number of germ cells (reviewed in Sharpe, 2001). During this phase, AMH and inhibin B are produced by the Sertoli cells, under the control of FSH, and are used as markers of proliferation and function, however their physiological role during "mini-puberty" is unknown (Crofton *et al.*, 2002; Aksglaede *et al.*, 2010; Kelsey *et al.*, 2016).

During the fetal and early postnatal period of development, centrally located gonocytes within the seminiferous cords differentiate and lose their pluripotency. At around two-three months of age, the differentiating cells migrate towards the basement membrane, developing into the SSCs (Huff *et al.*, 2001, reviewed in Culty, 2009; Sohni *et al.*, 2019). The SSCs are now located within the stem cell niche in the basal compartment of the seminiferous cords, outwith the blood-testis barrier (BTB) which will later form during puberty (reviewed in Stanton, 2016; Li *et al.*, 2017). The maintenance of the SSC niche is dependent on factors produced by testicular somatic cells, the basement membrane and vascular network within the interstitium. These factors control the SSCs behaviour to determine self-renewal, pluripotency, quiescence and differentiation of the SSCs (reviewed in De Rooij, 2017; Stukenborg *et al.*, 2018a). Differences in SSC development and dynamics between non-human primates and rodents are known, however to date the majority of research has been performed in rodent animal models. Signalling molecules involved in maintaining the SSC niche identified in rodent models include glial cell-derived neutrophic factor

(GDNF), basic fibroblast growth factor (bFGF), CXCL12 and WNT5A that are produced by Sertoli cells as well as macrophage colony stimulating factor (CSF1) and GDNF produced by PMCs (reviewed in de Rooij, 2009). In rodents, there is a relatively rapid and synchronous differentiation of gonocytes to pre-spermatogonia and migration to the basal membrane occurring by pnd six (Mitchell *et al.*, 2008; Wu *et al.*, 2009).

1.2.3 Prepuberty

Prepuberty in males lasts for around 11 years until the HPG axis is reactivated and the child enters puberty. Testicular activity during prepuberty was once considered low which would confer protection against chemotherapy treatment, since these agents particularly target proliferating cells (Rivkees and Crawford, 1988) (Fig 1.1). However, based on studies that have shown infertility in adulthood following chemotherapy treatment for childhood cancer, this would indicate important developmental processes occur during childhood (Chow *et al.*, 2016). Indeed, research has shown germ cell proliferation and Sertoli cell maturation, in particular, taking place during prepuberty (discussed below and reviewed in Chemes, (2001)).

During prepuberty, the testis volume is ~0.6ml (Joustra *et al.*, 2015). The proliferative rate of the germ cell population is reduced in comparison to fetal development, nevertheless there is a three-fold increase in the overall population during this time period (Müller and Skakkebaek, 1983). At this point in humans, the SSC pool is represented by undifferentiated A spermatogonia composed of two populations of cells, either with a dark (A_{dark}) or a pale (A_{pale}) appearance (reviewed in Stukenborg *et al.*, 2014; Fayomi and Orwig, 2018). A_{dark} spermatogonia are proposed to be the reserve stem cells with low mitotic activity, whilst A_{pale} cells actively proliferate and are considered the functional stem cell reserve, responsible for self-renewal of the SSC population. Upon differentiation, A_{pale} spermatogonia differentiate to B spermatogonia, which can be observed from four-to-five years of age (Paniagua and Nistal, 1984). A recent study investigating pubertal changes in the transcriptome and protein levels of prepubertal human testes samples have confirmed these

observations at the molecular level (Guo *et al.*, 2020). In rodents, undifferentiated spermatogonia are represented by A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia, with $A_{(s)}$ spermatogonia currently considered the functional SSC population which makes up 0.03% of the total germ cell population in the adult rodent testis (Huckins, 1971; Tagelenbosch and de Rooij, 1993; reviewed in Fayomi and Orwig, 2018). The A_s cells actively proliferate to form two conjoined daughter cells known as A_{pr} cells; these can either complete cytokinesis to produce two new A_s spermatogonia, thereby maintaining the stem cell pool, or continuously divide forming A_{al} chains (reviewed in de Rooij and Russell, 2000; Ehmcke *et al.*, 2006). This model of SSC proliferation is the most prevailing, having been described since the early 1970s (Huckins, 1971). Alternative models have been proposed, including the 'fragmentation' model whereby $A_{s,pr,al}$ spermatogonia are all considered functional SSCs, and the 'hierarchy' model whereby heterogeneity in the A_s population is proposed based on molecular studies (reviewd in de Rooij, 2017). This has led to research to identify the 'ultimate' SSC population, with research indicating a small, rare population of these cells in the rodent testis may exist outwith the population of $A_{s,pr,al}$ spermatogonia (reviewed in De Rooij, 2017; Helsel *et al.*, 2017). The A_{al} spermatogonia will continue to differentiate and undergo cellular divisions to form A_1, A_2, A_3, A_4, intermediate and type B spermatogonia that will divide to form primary spermatocytes that undergo meiosis (reviewed in Fayomi and Orwig, 2018). Type B spermatogonia are observed within the testis around pnd eight in mice and will eventually divide to form primary spermatocytes (Bellve *et al.*, 1977). Occasionally, spermatogonia enter meiosis, leading to the infrequent observation of primary spermatocytes during prepuberty, however these cells quickly degenerate, since the somatic cells are not mature enough to support full spermatogenesis at this stage (reviewed in Chemes, 2001). The diameter of the tubules is unaltered during prepubertal development, with the lumen expanding only later during puberty due to intense germ cell proliferation that results in expansion in tubule width (reviewed in Chemes, 2001). Reference values based upon a systematic review and meta-analysis performed by Masliukaite *et al.* (2016) indicates that spermatogonia number

per tubular cross section and density per area (cm^3) decrease during the first three years of life followed by a gradual increase up to six-to-seven years of age, plateauing up to age 11. The initial decrease may be a result of programmed cell death of the gonocytes that failed to migrate to the basement membrane earlier in development (Masliukaite *et al.*, 2016).

The Sertoli cells in the prepubertal testis appear morphologically immature, with little cytoplasm and with the nuclei arranged in palisade formation, with small nucleoli (reviewed in Chemes, 2001). Sertoli cells during prepuberty begin to mature gradually and asynchronously. The cells enlarge due to increased cytoplasmic volume, and form hemidesmosome-like unions with the basal lamina forming the scaffolding of the seminiferous epithelium. This is essential to support spermatogenesis in adulthood (Rey *et al.*, 1993; reviewed in Edelsztein and Rey, 2019; Guo *et al.*, 2020). These somatic cells proliferate to an extent during puberty, although at a reduced rate in comparison to the proliferative activity of the germ cells during prepuberty (Cortes *et al.*, 1987). The Sertoli cells undergo functional maturation with progressive increased expression of androgen receptors and connexin 43, as well as expression of vimentin and inhibin B (Brehm *et al.*, 2006; Kelsey *et al.*, 2016; De Michele *et al.*, 2018). In addition, Sertoli cells display aromatase activity and produce oestrogen during prepuberty (reviewed in Chemes, 2001). AMH, in particular, is secreted in large amounts in prepubertal boys and can be used as an indicator of Sertoli cell number and function (reviewed in Rey, 1999). As in the fetus, Sertoli cell factors are involved in controlling the development/proliferation of other testicular cell types. The development of the PMCs and adult Leydig cells during the prepubertal period is less well understood with much of our knowledge based on rodent studies (Chen *et al.*, 2009; Rebourcet *et al.*, 2014). The PMCs proliferate and develop during early postnatal development in the rodent, under the control of FSH, with Sertoli cells having an important role in maintaining their differentiated state (reviewed in Chemes, 2001; Nurmio *et al.*, 2012; Rebourcet *et al.*, 2014). However, this dependency is lost when the PMCs terminally differentiate during prepuberty, as

shown in the rodent model (Rebourcet *et al.*, 2014). In the rodent model, mesenchymal-like cells that will eventually form the ALCs reside within the interstitium. Sertoli cells have an important role in stimulating ALC differentiation and will ultimately determine the number of ALCs in the adult (reviewed in Chen *et al.*, 2009; Rebourcet *et al.*, 2014). The Guo *et al.* (2020) study has shed some light on human development during prepuberty, identifying a common progenitor population of Leydig cells and PMCs based on co-expression of key markers of immature and mature Leydig cells and PMCs.

1.2.4 Puberty/Adulthood

Timing of entry into puberty varies between children and is dependent on genetic and environmental factors; on average boys enter between 10 and 13 years of age. Boys are considered to have entered puberty, clinically, upon reaching testicular volume of 4ml as a result of extensive germ cell proliferation, formation of meiotic germ cells and expansion of tubule width (reviewed in Stukenborg *et al.*, 2010). The Masliukaite *et al.*, (2016) study has shown dramatic increases in the number of germ cells from as early as 11 years of age, which coincides with the onset of puberty. During the mid/late phase of prepuberty, the HPG axis is reactivated, producing gonadotrophins FSH and LH in a pulsatile manner nocturnally (reviewed in Herbison, 2016; Wood *et al.*, 2019). These hormones are produced by the anterior pituitary and are essential for stimulation of spermatogenesis and production of testosterone (Fig 1.2). The production of FSH is key during late pre-puberty/early puberty to stimulate proliferation of the germ cell and Sertoli cell populations, while LH results in the formation of the ALCs which produce testosterone through steriodogenesis (Ma *et al.*, 2004; reviewed in Chen *et al.*, 2009; Plant, 2015; Koskenniemi *et al.*, 2017).

Testosterone is essential for testis development and function, with androgen receptors expressed on Sertoil cells, Leydig cells and PMCs (Shan *et al.*, 1995; Welsh *et al.*, 2009; O'Shaughnessy *et al.*, 2012, 2019; reviewed in O'Hara and Smith, 2015 & Edelsztein and Rey, 2019). Levels of AMH decline during puberty, as testosterone

levels increase due to action on the Sertoli cells, which express high levels of androgen receptors by this stage of development. Inhibin B concentrations increase during puberty, with secretion from Sertoli cells stimulated through negative feedback loop with FSH, and peak at around 17 years of age before reaching adult levels (Kelsey *et al.*, 2016). Levels of inhibin B are indicative of spermatogenesis and are being investigated as a potential marker for Sertoli cell function (reviewed in Anderson and Sharpe, 2000). Sertoli cells cease to proliferate and will mature as they enter puberty (Sharpe *et al.*, 2003; Guo *et al.*, 2020). This maturation triggers formation of the blood testis barrier (BTB) which divides the seminiferous tubule into a basal compartment (containing SSCs, B spermatogonia and preleptotene spermatocytes) and an adluminal compartment where meiotic cells traverse an immune-privileged environment (reviewed in Yan Cheng and Mruk, 2012). The BTB forms from intercellular junctions between adjacent Sertoli cells near the basement membrane and is composed of tight junctions, adherens junctions (testis specific, basal ectoplasmic specializations), gap junctions and desmosomes. The BTB is a dynamic structure that disassembles to allow preleptotene spermatocytes to enter the adluminal compartment and then reassembles. The formation of the BTB in rodents takes place from pnd 15 to 21 (Vitale *et al.*, 1973). A well-defined basal lamina forms during puberty through the action of somatic cells, particularly the PMCs, which occurs as spermatogonia differentiate (Guo *et al.*, 2020). The PMCs differentiate during puberty and gain the ability to contract, with testosterone stimulating the expression of smooth muscle actin (reviewed in Mayerhofer, 2013). Testosterone action on the Sertoli cells also initiates meiotic progression of the germ cell population, although the underlying mechanism is unknown (Guo *et al.*, 2020). Edelsztein and Rey, (2019) propose that changes in cytoskeletal structure of the Sertoli cells induces a corresponding change in the germ cell population which initiates meiosis and enables these cells to pass through the BTB. Once in the adluminal compartment, the preloptene spermatocytes progress through the rest of meiosis, through lepotene, zygotene, pachytene and diplotene stages, before completing the second round of meiotic divisions and forming haploid spermatids.

The round spermatids develop into elongated spermatozoa during spermiogenesis where the cells undergo morphological differentiation (Clermont, 1972; reviewed in Ehmcke *et al.*, 2006; & Stukenborg *et al.*, 2014). Spermatogenesis takes 74 days in humans and 34.5 days in mice (commencing ~pnd35). Mice produce 10-fold higher yields of sperm per gram of testis tissue than humans since spermatogenesis in the mouse has more transit-amplification steps i.e. there are a greater number of cellular divisions of spermatogonia leading to a higher yield of sperm derived from a single SSC in rodents compared to humans (Clermont, 1972; reviewed in Fayomi and Orwig, 2018). Overall, humans produce 4.4 million sperm per gram of testicular parenchyma per day, compared to mice who produce ~40 million sperm per gram of testicular parenchyma per day (Vergouwen *et al.*, 1993; reviewed in Fayomi and Orwig, 2018).

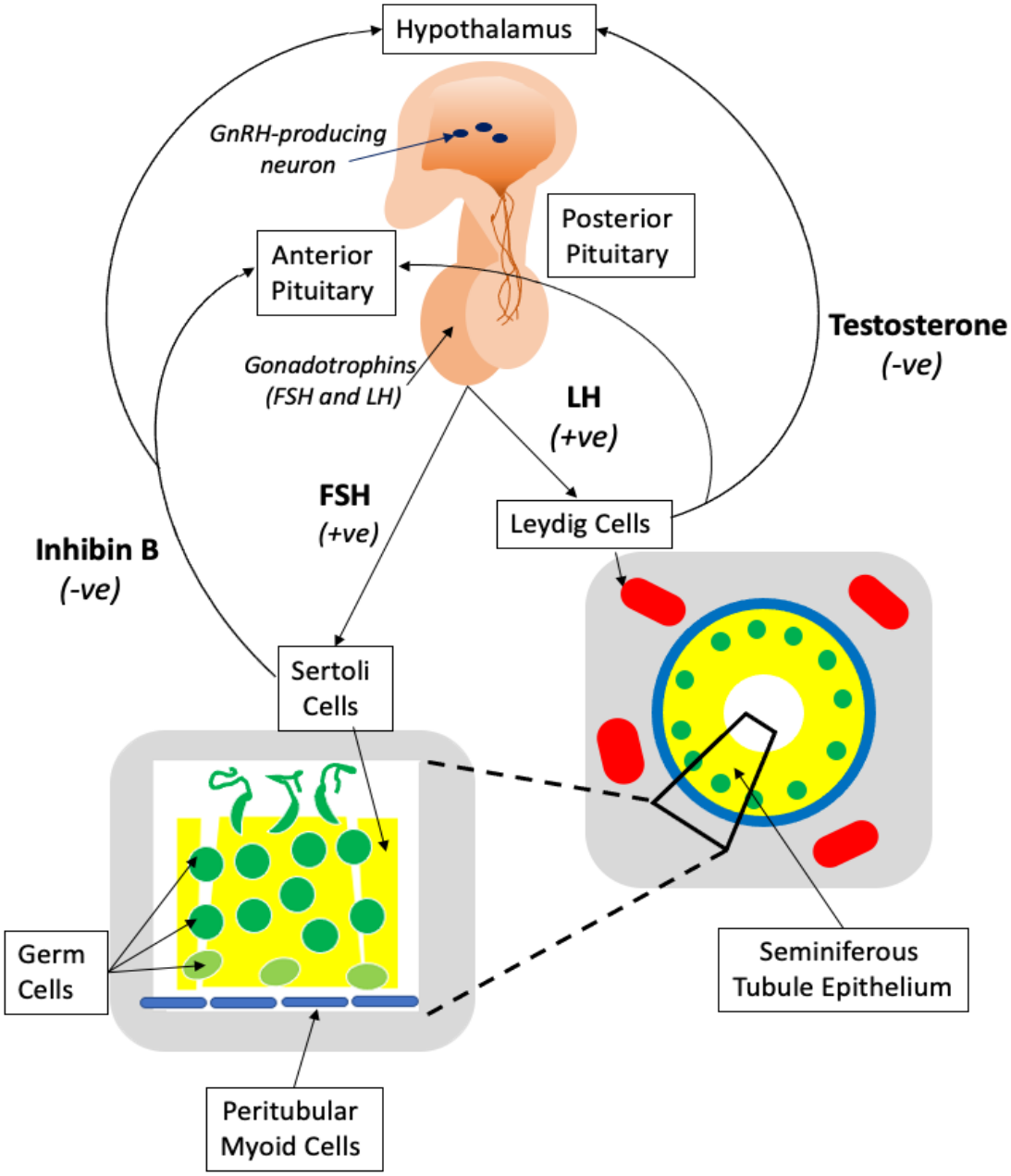


Figure 1.2: Overview of the HPG axis in the adult male. The gonadotrophins FSH and LH are produced from the anterior pituitary under control of gonadotrophin releasing hormone (GnRH) produced by the hypothalamus. FSH acts upon the Sertoli cell population within the seminiferous tubules stimulating spermatogenesis and producing inhibin B. LH acts upon the Leydig cell population within the interstitium stimulating production of testosterone that acts upon the Sertoli cell to regulate spermatogenesis. Inhibin B and testosterone provide negative feedback to the hypothalamus and anterior pituitary to control GnRH and gonadotrophin secretions. Testosterone can be aromatised into oestradiol and dihydrotestosterone which can also provide negative feedback. Image modified from Llahana *et al.* (2019).

1.3 Childhood Cancer and Treatment

1.3.1 Childhood Cancer

Each year, around 1900 children (ages 0 to 14 years) are diagnosed with cancer, accounting for 1% of new cancer diagnoses in the UK (reviewed in Wallace, 2011; Cancer Research UK, 2020; Children with Cancer UK, 2020). Cancer is the second most common cause of death in children. The range of cancers that develop at this young age differ from those of adult malignant tumours. In infants, tumours of embryonal origin such as neuroblastomas are the most frequent cause, whereas in older children leukaemia (particularly acute lymphoblastic leukaemia) as well as central nervous system tumours and lymphomas (non-Hodgkins and Hodgkins) are more often diagnosed. There is also a sex difference in incidence, with boys 1.2 times more likely to be diagnosed with cancer, particularly lymphomas and central nervous system tumours (reviewed in Kaatsch, 2010; Kelly, 2017). The number of children surviving childhood cancer has substantially increased in the last few decades, with survival rates of five years or more around 80% (Miller *et al.*, 2016). In the UK to date, there is an estimated 35,000 survivors of childhood cancer, with this number considered likely to grow by around 1300 per year (Children with Cancer UK, 2020).

The impact of cancer treatments, including radiotherapy, chemotherapy and immunotherapy, can reduce the quality of life of childhood cancer survivors as they age (reviewed in Robison and Hudson, 2014; Landier *et al.*, 2015, 2018). Based on a self-reporting study of co-morbidities following childhood cancer treatment (Oeffinger *et al.*, 2006), it is estimated that around 70% of survivors 30 years after diagnosis were living with chronic health conditions, such as cardiovascular, musculoskeletal, endocrinological and renal disorders. In addition, 40% suffered from severe health problems including congestive heart failure, myocardial infarction, cognitive dysfunction and premature gonadal failure. When assessed clinically, it was determined that up to 98-99% of survivors were suffering from one or more chronic health conditions including; pulmonary disease, spinal disorders and secondary cancers, and 68% with severe health conditions 10 to 50 years after diagnosis

(Hudson *et al.*, 2013; Bhakta *et al.*, 2017). Childhood cancer survivors are also at an increased risk of developing psychological disorders such as anxiety and depression, delayed psychosexual development and are less likely to be married or live with a partner (reviewed in Brinkman *et al.*, 2018). These psychosocial factors could account for observed reduced fertility in studies such as Chow *et al.* (2016), where there was a reduced fertility rate between male survivors of childhood cancer and siblings controls of around 25%. For example, when comparing never married status of childhood cancer survivors and siblings for males, there is a 14% difference (Janson *et al.*, 2009).

1.3.2 Chemotherapy Treatment

Chemotherapy and radiotherapy are a mainstay of treatment for childhood malignancies. For chemotherapy drugs, the refinement and use of combined drug treatments have contributed greatly to the dramatic increase in childhood cancer survival rates over recent decades (reviewed in Anderson *et al.*, 2015; Miller *et al.*, 2016). Treatment with certain chemotherapy agents, however, are associated with adverse health outcomes in adulthood (Table 1) (reviewed in Hudson *et al.*, 2013; Robison and Hudson, 2014) Multiple different drugs have been established in treatment regimens; these kill cancer cells through various mechanisms, often targeting proliferating cells (Table 1) (reviewed in Malhotra and Perry, 2003; Lind, 2011). The gonadotoxic impact and relative risk of later infertility of individual chemotherapy agents included in treatment regimens against childhood malignancies can at present only be estimated, since the risk is dependent on multiple factors including dosage, treatment length, age at time of treatment and the sensitivity of individual patients to chemotherapy treatment. The current classification of chemotherapy drugs, and the dosages considered to result in infertility, are outlined in Table 1.1 (Meirow and Schenker, 1995; Howell and Shalet, 2001; Wyns *et al.*, 2010; Tournaye *et al.*, 2014; Lambertini *et al.*, 2016). However, these classifications/dosages are debated. Given that clinicians use this knowledge to determine whether or not to offer cryopreservation of testicular tissue before

commencement of treatment, further elucidation on relative risk/gonadotoxicity is urgently required (reviewed in Goossens *et al.*, 2020). At present, alkylating and alkylating-like agents such as cyclophosphamide and cisplatin, drugs that intercalate into DNA, disrupting basic cellular processes, are considered to be highly gonadotoxic. These agents are commonly included in treatment regimens to treat a wide range of childhood malignancies, and are known to result in subsequent infertility in adulthood (Chow *et al.*, 2016). Agents from alternative drug classes which have different mechanisms of action are considered low or moderate risk of later infertility and include; anti-metabolites such as cytarabine, vinca alkaloids e.g. vincristine and topoisomerase inhibitors including etoposide and doxorubicin. There are also some chemotherapy agents, including taxanes, where infertility risk/gonadotoxicity is at present unknown. Further details are included in Table 1. During this PhD, the chemotherapy agents cisplatin, doxorubicin and vincristine were chosen to further investigate gonadotoxicity, the underlying mechanism action and the potential of antioxidants to protect against chemotherapy-induced gonadotoxicity.

For the vast majority of paediatric cancers, combined chemotherapy with multiple agents is required to effectively treat the disease, with commonly used combinations including MOPP (nitrogen mustard, vincristine, procarbazine and prednisolone) or ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) for treatment of Hodgkins lymphoma and CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) for Non-Hodgkins lymphoma (reviewed in Corrie, 2011). The administration of several agents in a treatment regimen could result in complex interactions of the drugs, for example potentially resulting in additive or even multiplicative side effects on healthy tissues. In theory, such agents could also cancel out the effects of other agents.

Table 1.1: Overview of commonly used chemotherapy agents in childhood cancer treatment regimens. Updated since publication in Allen *et al.* (2018)

Chemotherapy Drug Class	Childhood Cancer Usage	Mechanism of Action	Cell Cycle Phase	Compounds	Current prediction of Infertility Risk *	Known Co-Morbidities in Adulthood**
Alkylating and Alkylating-Like Agents	Hodgkins Lymphoma Non-Hodgkins Lymphoma Leukemia CNS Tumors Kidney Cancer Neuroblastoma Soft Tissue Sarcoma Bone Cancer	Alkyl groups intercalate into nucleic acids and proteins. Intercalate into DNA by binding to the guanine or cytosine bases, resulting in crosslinks which disrupt DNA replication/transcription.	Non-specific	Ifosfamide Cyclophosphamide Procarbazine Chlorambucil Melphalan Cisplatin Mechlorethamine Carboplatin Oxaliplatin Busulfan Carmustine Lomustine	High (>60g/m^2) High (>7.5g/m^2) High (>4g/m^2) High (>1.4g/m^2) High (0.14g/m^2) High (>0.6g/m^2) High Moderate Moderate	Pulmonary fibrosis (busulfan, carmustine and lomustine) Urinary tract abnormalities (cyclophosphamide and ifosfamide) Renal dysfunction (cisplatin, carboplatin and ifosfamide) Ototoxicity (cisplatin) Dyslipidemia (cisplatin)
Anti-Metabolites	Leukemia	Disrupt DNA/RNA synthesis	S-phase	Cytarabine Methotrexate	Moderate Low	Neurocognitive impairment

	Non-Hodgkins Lymphoma Bone Cancer			Mercaptopurine Fluorouracil Thioguanine	Low Unknown Unknown	Leukoencephalopathy Liver dysfunction Renal toxicity Reduced bone mineral density
Vinca Alkaloids	Hodgkins Lymphoma Non-Hodgkins Lymphoma Leukemia CNS Tumor Kidney Cancer Neuroblastoma Soft Tissue Sarcoma Bone Cancer	Inhibit assembly of microtubules	M-phase	Vincristine Vinblastine	Low Low	Peripheral sensory and motor neuropathy
Taxanes	Ewing Sarcoma	Inhibit disassembly of microtubules	G_2/M-interphase	Paclitaxel Docetaxel	Unknown Unknown	Neurotoxicity Muscle and Joint Pain Skin conditions Myelosuppression
Topoisomerase Inhibitors	Leukemia Soft Tissue Sarcoma Bone Cancer	Target either topoisomerase I or II to disrupt DNA replication	M or S-phase	Etoposide Irinotecan Topotecan Teniposide	Low Unknown Unknown Unknown	Acute myeloid leukemia

Anthracyclines	Hodgkins Lymphoma Non-Hodgkins Lymphoma Leukemia Kidney Cancer Neuroblastoma Soft Tissue Sarcoma Bone Cancer	Target topoisomerase II, intercalate into DNA and producing free radicals	M-phase	Doxorubicin Daunorubicin	Moderate Unknown	Cardiomyopathies Dysrythmie Left ventricular dysfunction
Antitumor Antibiotics	Hodgkins Lymphoma Soft Tissue Sarcoma Bone Cancer	Multiple mechanisms of action including DNA intercalation, disruption of DNA synthesis and DNA fragmentation.	Non-specific	Bleomycin Dactinomycin Mitomycin C	Low Low	Lung fibrosis (bleomycin) Thrombocytopenia and leukocytopenia (Mitomycin C)

*: Currently considered risk of infertility adapted from Wyns *et al.*, (2010). ** from (Verweij and Pinedo, 1990; Mir *et al.*, 1996; Cella *et al.*, 2003; Robison and Hudson, 2014).

1.4 Clinical Studies investigating Chemotherapy-induced Damage in Prepubertal Patients or Adult Survivors

Understanding the underlying mechanisms of action and molecular targets of different classes of chemotherapy drugs that damage the prepubertal testis is essential to aid development of cytoprotective agents. Chemotherapy-induced damage to the reproductive tissues can adversely impact on reproductive outcomes in adulthood, including impaired development of sexual characteristics and infertility (Frederick *et al.*, 2016). Long-term fertility of individuals depends on the survival and health of the germ cell population, specifically SSCs, and somatic cells (Zohni *et al.*, 2012; Yoon *et al.*, 2017; Stukenborg *et al.*, 2018a). Research focusing on direct damage to the testis within a clinical setting is lacking, since testis tissue biopsy is not routinely performed before or after chemotherapy treatment. Often, indirect methods are used to assess chemotherapy-induced damage, including assessment of levels of gonadotrophins and semen analysis. Assessment of damage to the prepubertal testis can be assessed during/after childhood cancer treatment or implied from subsequent analysis of the adult testis (reviewed in Kelsey *et al.*, 2017; Skinner *et al.*, 2017). With recent focus on cryopreserving prepubertal testis samples before the onset of cytotoxic treatment for potential fertility preservation in the future, more prepubertal tissue is becoming available for research and therefore studies using such tissue should be more common in the future (Valli-Pulaski *et al.*, 2019; Portela *et al.*, 2020). Indeed, a report by Stukenborg *et al.* (2018b) has histologically examined testis tissue biopsies from prepubertal patients who were selected for cryopreservation of tissue due to cytotoxic nature of their cancer treatment regimens. Cryopreservation of testis tissue is recommended before the start of cancer treatment, however indications from the Valli-Pulaski *et al.* (2019) study show that early chemotherapy treatment, including alkylating agents, may not significantly affect the number of undifferentiated spermatogonia in the biopsy.

Studies examining the direct testicular damage, through for example analysis of morphology, following chemotherapy treatment of prepubertal human patients are

rare. To date, the majority of studies have associated the damage to cyclophosphamide treatment, although in the majority of cases there is co-administration of other chemotherapy drugs (Table 1.2). Methods of analysis are descriptive in nature, following histological analysis of testicular biopsies, for example describing the general appearance of tubules and density of germ and somatic cells present. Additional analyses include calculation of the tubular fertility index (TFI), which represents the percentage of seminiferous tubules that contain spermatogonia (Ise *et al.*, 1986). Many of the included studies present limitations such as small patient cohorts, lack of universal methods for defining pubertal status, and lack of adequate control groups for comparison.

1.4.1 Assessment of Immediate Testicular Damage

Initial histological observations from early case reports (Arneil, 1972; Berry *et al.*, 1972; Hyman and Gilbert, 1972) produced inconsistent results as to whether chemotherapy treatments damage the prepubertal testis. Later, larger cohort studies clearly show a relationship between use of alkylating agents in treatment regimens and testicular tissue damage. In particular, the inclusion of the drug cyclophosphamide for cancer treatment during the prepubertal period has been linked to resulting testicular damage (Table 1.2A). The studies included here under 'immediate assessment' varied in the timeframe of analysis, with the testicular damage examined at different time points including during, just before the cessation of, or at the end of the treatment period, as well as up to a year after the end of treatment. These investigations have indicated that cyclophosphamide treatment is associated with testicular damage in a dose- and time-dependent manner. Treatment can reduce the overall size of the testis where there is depletion of the germ cell population, resulting in Sertoli cell-only tubules, as well as interstitial fibrosis and basement membrane thickening (Hyman and Gilbert, 1972; Hensle *et al.*, 1984; Uderzo *et al.*, 1984). A threshold dose at which such damage is evident is hard to define, since comparison between the few available studies is challenging due to the limitations previously described, as well as the variability of treatment regimens. The

length of the treatment regimens may also determine the severity of the impairment, with higher cumulative doses over a shorter period of time reducing chemotherapy-induced damage (e.g. Ise *et al.*, 1986). Indications of increased number of germ cells in testis biopsies of prepubertal boys 18-63 days after chemotherapy treatment have been described in Van Saen *et al.* (2015). Although these results were not significantly different to an untreated control group, the researchers do suggest that this may showcase the potential for germ cell replenishment by action of the reserve stem cell population within the testis, the A_{dark} spermatogonia. In many of the studies listed in Table 1.2 and discussed further in subsequent sections, only a subset of the patients exhibited severe damage to the testis following cyclophosphamide treatment. This indicates a degree of variability/susceptibility to damage, which could be due to several factors, including age and genetic predisposition.

The testis itself is composed of somatic and germ cells, which could potentially each have different sensitivities to chemotherapeutic drugs. Damage to the somatic cells during chemotherapy treatment could negatively impact on the germ cells and vice versa (reviewed in Stukenborg *et al.*, 2018a). Nurmio *et al.*, (2009a) have reported that cyclophosphamide targets both SSCs and more differentiated spermatogonia in the prepubertal testes, as indicated by changes in gene expression of specific spermatogonial markers (MAGE A4 and CD9). This is in agreement with most papers that reported effects on germ cells, with one study describing the appearance of immature Leydig and Sertoli cells following cyclophosphamide treatment based on morphological examination (Ise *et al.*, 1986). This suggests treatment may interfere with the development/maturation of the somatic cell populations.

Table 1.2A: Human studies reporting cyclophosphamide-induced gonadotoxicity: assessment of immediate testicular damage. Updated since published in (Allen *et al.*, 2018)

Cyclophosphamide Dosage		Other drugs co-administered	Treatment Length	Number of Male Patients within the Study & Age (yrs)	Effect of Cyclophosphamide				Papers
					Testicular Histology	Number of germ or somatic cells/ TFI	Interstitial Fibrosis/Base-ment membrane thickening	Testicular Size	
Grams per Body Weight	0.002g/kg /day	Unknown	3 mths	Patients: 1 Prepubertal: 1 CYC treated: 1 Age: 6	Abnormal, atrophic tubules	SCOs	Present	Small	Hyman and Gilbert, 1972
	0.003-0.024g/kg /day	Unknown	<50-400 days	Patients: 7 Prepubertal: 7	Normal	N/A	N/A	N/A	Arneil, 1972

Grams per Body Weight				CYC treated: 7 Age: 3-11					
	0.475-0.846g/kg	Vincristine Prednisone Asparaginse Cytarabine Methotrexate Mercaptopurine Doxorubicin	2-6 yrs	Patients: 46 Prepubertal: # CYC treated: # Age: 0.08-13	Abnormal	Reduced TFI >0.130g/kg CYC. Normal TFI intermittently treated higher cumulative CYC. Poorly developed somatic cells younger patients	N/A	N/A	Ise *et al.*, 1986
Grams per Area	0.12-8.5 g/m^2	Nitrogen Mustard Dacarbazine Lomustine Carmustine Procarbazine Methotrexate Mercaptopurine Fluorouracil Doxorubicin	0.08 – 1 yr	Patients: 32 Prepubertal: 21 CYC treated: 13 Age: <11	Abnormal when treated with multiagent regimen including CYC	N/A	N/A	N/A	Matus-Ridley *et al.*, 1985

Grams per Area		Vincristine Vinblastine Vindesine Prednisone Asparaginase Teniposide Daunorubicin Dactinomycin Bleomycin Mitomycin C Cytarabine							
	0.5 – 1.2g/m^2	Vincristine Prednisone Mercaptopurine Methotrexate Asparaginase Daunorubicin Doxorubicin Cytarabine	1.2-88.8 mths	Patients: 10 Prepubertal: 8 CYC treated: 2 Age: 9.4-16.6	N/A	Complete loss of GCs CYC treated	N/A	N/A	Müller *et al.*, 1985
	>1g/m^2	Vincristine Prednisone Mercaptopurine Methotrexate Asparaginase	12-77 mths	Patients: 44 Prepubertal: 27 CYC treated: # Age: 3.5-15	N/A	Reduced TFI (<40%) >1g/m^2 CYC	Present	N/A	Lendon *et al.*, 1979

Grams per Area		Doxorubicin Cytarabine							
	3-16g/m^2	Vincristine Doxorubicin Asparaginase Cytarabine Mercaptopurine Methotrexate Thioguanine Prednisone Dexamethasone	~1.8 yrs	Patients: 37 Prepubertal: 37 CYC treated: 16 Age: 1.1-16.1	N/A	Depletion of spermatogonial pool with reduced TFI (19%)	N/A	N/A	Poganitsch-Korhonen *et al.*, 2017
	<4.8g/m^2	Asparaginase Daunorubicin Hydroxyurea Lomustine Methotrexate Prenisolone Thioguanine Vincristine Cytarabine	3-4 yrs	Patients: 25 Prepubertal: 24 CYC treated: 24 Age: 1.23-12.35	Abnormal	Complete loss or depletion of GC pool. Normal SC	N/A	Small	Quigley *et al.*, 1989

Grams per Area	6.2-11.4g/m^2	Prednisone Vincristine Doxorubicin Methotrexate Asparaginase Cytarabine Teniposide Mercaptopurine	6.6 -7.6 yrs	Patients: 23 Prepubertal: 23 CYC treated: 6 Age: 2.8-8.6	N/A	Depletion in SSCs (↓CD9 & OCT4) and more differentiated spermatogonia (↓ MAGE4). Recovery noted	N/A	N/A	Nurmio *et al.*, 2009a
	8-16.8g/m^2	Cytarabine Vincristine Prednisone Methotrexate Asparaginase Dexamethasone Enocitabine Mercaptopurine Daunorubicin Hydrocortisone	Unknown	Patients: 12 Prepubertal: # CYC treated: 7 Age: 1-12	N/A	Reduced TFI (<50%) with morphological changes to GCs and SCs but not linked to CYC	Present	N/A	Kobayashi *et al.*, 1996
	2-4200g/m^2	Etoposide Mitoxantrone Cytarabine Cisplatin Carboplatin	Unknown	Patients: 7 Prepubertal: # CYC treated: 2	N/A	GC markers ↑ (MAGEA4 or UCHLI) per cell and per tubule. Not	N/A	N/A	Van Saen *et al.*, 2015

Grams per Area		Vincristine Daunorubicin Asparaginase Doxorubicin Ifosfamide Methotrexate Idarubicin Thioguanine		Age: 3-15		statistically significant. ↓NANOG (pluripotency) in 12yr old patient (CYC-treated) Similar SC and LC marker expression as controls.			
Total Dose	1.4-20.8g	Vincristine Prednisone Mercaptopurine Methotrexate Asparaginase Cytarabine Doxorubicin	~4.4 yrs	Patients: 37 Prepubertal: # CYC treated: 14 Age: 1.6-14.3	Abnormal	GC damage and reduced TFI (<50%)	N/A	N/A	Wallace *et al.*, 1991

Total Dose	1.4-11.8g	Vincristine Prednisone Asparaginase Methotrexate Mercaptopurine Cytarabine Adriamycin	30-81 mths	Patients: 35 Prepubertal: 29 CYC treated: 5 Age: 4.9-13.7	Abnormal	Reduced TFI (<30%) when treated with CYC	Basement membrane thickening present	N/A	Uderzo *et al.*, 1984
	7.79g	Prednisone	180 days	Patients: 1 Prepubertal: 1 CYC treated: 1 Age: 3-4	Normal	N/A	N/A	Normal	Berry *et al.*, 1972

CYC; cyclophosphamide, GC: germ cells, LC; Leydig cells, mths; months, yrs; years, TFI; tubular fertility index, SCO; Sertoli cell only tubules, SC; Sertoli cells, SSC; spermatogonial stem cells. # Information not included in study

1.4.2 Assessment of the effect of Prepubertal Drug Exposure Damage in the Adult

Insult to the prepubertal testes following chemotherapy treatment can be determined through examination of the potential for the testes to recover and later undergo active spermatogenesis; this can be inferred from examination of pubertal/adult patients who were treated as children. Short-term analysis (one-to-five years) and long-term analysis (six-to-ten years) following the cessation of cyclophosphamide treatment have shown that damage is often still observable in a dose- and time- dependent manner (Table 1.2B). Patients receiving relatively high doses of cyclophosphamide have exhibited severe testicular damage with Sertoli cell only tubules present up to nine years after treatment (Penso *et al.*, 1974; Aubier *et al.*, 1989). The length of treatment regimen may also influence the disruption caused to the prepubertal testes, as shown in Etteldorf *et al.* (1976) (Table 1.2B). However, such differences may ultimately be the result of higher cumulative doses or the age of the patient when treated, with younger patients potentially more at risk of reduced TFI and poor development of Sertoli and Leydig cells, as has been shown in Ise *et al.* (1986). Nonetheless, due to small numbers of participants, individual studies such as this can only lead to definitive conclusions when part of a larger meta-analysis. A case report has described somatic cell damage in the testis following chemotherapy during prepuberty, with the presence of immature Sertoli cells (identified by cytokeratin 18 & D2-40 markers) in a 31-year-old man treated during childhood with a regimen containing cyclophosphamide; however causation cannot be determined from a case report (Bar-Shira Maymon *et al.*, 2004). Overview of direct damage to the testis as assessed in the adult shown in Table 1.2B.

Table 1.2B Human studies reporting cyclophosphamide-induced gonadotoxicity: subsequent assessment in the adult. As published in Allen *et al.* (2018)

Cyclophosphamide Dosage		Other drugs co-administered	Treatment Length	Number of Patients within the Study & Age (yrs)	Effect		Papers
					Short-term (1-5yrs)	Long-term(6-10yrs)	
Grams per Body Weight	0.2-0.5g/kg/day	Unknown	1.5 – 6 mths	Patients: 23 Prepubertal: 16 CYC treated: 23 Age: 3.5-20	Abnormal histology 20-25% of tubules atrophic high cumulative doses	N/A	Pennisi *et al.*, 1975
	0.104- 0.2g/kg	Prednisone	49-60 days	Patients: 4 Prepubertal: # CYC treated:4 Age: 9-13	N/A	Normal testis morphology	Etteldorf *et al.*, 1976
	0.312 – 1.325g/kg		89-489 days	Patients: 4 Prepubertal: # CYC treated: 4 Age: 5-8.5	N/A	Damage to seminiferous epithelium with SCOs	
	0.475-0.846g/kg	Vincristine Doxorubicin Asparaginase Cytarabine	2 to 6 yrs	Patients: 46 Prepubertal: # CYC treated: # Age: 0.08-13	Tubular damage occasionally observed four years after	N/A	Ise *et al.*, 1986

		Methotrexate Mercaptopurine Prenisone			treatment with unknown “low” doses		
Grams per Area	2.6-29g/m^2	Dactinomycin Doxorubicin Daunomycin Mercaptopurine Asparaginase Procarbazine Mechlorethamine Fluorouracil Cytarabine Vincristine	Unknown	Patients: 30 Prepubertal: 19 CYC treated: 10 Age: 1.75-17	N/A	Abnormal morphology	Aubier *et al.*, 1989
	2.75-7.5g/m2	Vincristine Prednisone Asparaginase Methotrexate Mercaptopurine Doxorubicin Cytosine arabinoside Carmustine Thioguanine	3- 4 yrs	Patients: 17 Prepubertal:17 CYC treated: 5 Age: 2.5-12.4	Reduced number of GC per mm3	N/A	Müller *et al.*, 1988

Total Dose	0.021 to 39g	Mercaptopurine Prednisone Fluoxymesterone Oxandrolone	9 wks -19 mths	Patients: 7 Prepubertal: # CYC treated: 7 Age: 11-16	High doses; SCOs, peritubular fibrosis, normal morphology LCs. Low doses; active spermatogenesis 90% of tubules.	N/A	Penso *et al.*, 1974

CYC; cyclophosphamide, GC: germ cells, LC; Leydig cells, mths; months, yrs; years, TFI; tubular fertility index, SCO; Sertoli cell only tubules, SC; Sertoli cells, SSC; spermatogonial stem cells. # Information not included in study

Rather than direct measurement of damage from testis biopsies, a marker to assess levels of damage would provide a less invasive indicator of chemotherapy-induced damage. Such markers include the assessment of the patients/survivors hormonal profile, pubertal status and semen parameters (reviewed in Kelsey *et al.*, 2017; Skinner *et al.*, 2017; Tian En *et al.*, 2020). Studies in survivors have measured levels of hormones in the blood as an indicator of gonadal damage and have found in a subset of survivors increased levels of basal and stimulated FSH and LH with reduced inhibin B and either normal or reduced testosterone concentrations (Ahmed *et al.*, 1983; Bramswig *et al.*, 1990; Wallace *et al.*, 1991; Heikens *et al.*, 1996; Mackie *et al.*, 1996; Müller *et al.*, 1996; Gerres *et al.*, 1998; Cicognani *et al.*, 2000; Gerl *et al.*, 2001; Kenney *et al.*, 2001; Rafsanjani *et al.*, 2007; Romerius *et al.*, 2009; van Casteren *et al.*, 2009; Tromp *et al.*, 2011; Isaksson *et al.*, 2018). Observations were found to correlate specifically with dose of alkylating agents, for example cyclophosamide and/or procarbazine (Bramswig *et al.*, 1990; Gerres *et al.*, 1998; Kenney *et al.*, 2001; van Casteren *et al.*, 2009; Jahnukainen *et al.*, 2011; Tromp *et al.*, 2011), as well as high cumulative doses of cisplatin (Wallace *et al.*, 1989; Gerl *et al.*, 2001; reviewed in Tian En *et al.*, 2020). The vast majority of these childhood cancer survivors pass through puberty normally, however, some patients have reported delayed entry into puberty and reduced testis volume (Wallace *et al.*, 1989, 1991; Bramswig *et al.*, 1990; Heikens *et al.*, 1996; Mackie *et al.*, 1996). The overall conclusions drawn from these studies suggests the occurrence of Sertoli cell and Leydig cell dysfunction resulting in impaired spermatogenesis in a subset of childhood cancer survivors, possibly dependent on cumulative doses, age/pubertal status at time of treatment and/or patient sensitivity. Inhibin B has been proposed as a good marker for gonadal damage following chemotherapy treatment, where reduced levels indicate reduced spermatogenesis (Cicognani *et al.*, 2000; Bordallo *et al.*, 2004; Stewart and Turner, 2005; Van Beek *et al.*, 2007). Analysis of semen parameters in the adult is currently considered the gold standard measurement for gonadal dysfunction after chemotherapy treatment (reviewed in Skinner *et al.*, 2017). Indeed, several studies have reported increased incidences of azoospermia and oligospermia following

chemotherapy treatment in childhood, particularly when exposed to alkylating agents (Bramswig *et al.*, 1990; Wallace *et al.*, 1991; Heikens *et al.*, 1996; Müller *et al.*, 1996; Kenney *et al.*, 2001; Longhi *et al.*, 2003; Bordallo *et al.*, 2004; Rafsanjani *et al.*, 2007; van Casteren *et al.*, 2009; Green *et al.*, 2010; Romerius *et al.*, 2011). The St Judes Lifetime Cohort study, which follows adult survivors of childhood cancer, have shown that of 214 survivors analysed; 25% were azoospermic, 28% oligospermic and 48% normospermic up to 21 years after diagnosis. This was significantly correlated with higher cyclophosphamide equivalent doses (CED), which estimates the cumulative dose of all alkylating agents received (Green *et al.*, 2014). Many survivors of childhood cancer have reported reduced fertility in adulthood (reviewed in Brougham and Wallace, 2005; Wasilewski-Masker *et al.*, 2014; Chow *et al.*, 2016). Fertility was reduced ~25% in comparison to siblings as controls with significant correlations noted for cyclophasmide, procarbazine, ifosfamide and bleomycin treatment (Wasilewski-Masker *et al.*, 2014; Chow *et al.*, 2016). Certain combinations of chemotherapy agents that include alkylating drugs within them, such as the MOPP combination used to treat Hodgkin's lymphoma, are known to damage the testis and result in azoospermia. However, alternative combinations that have been proven to be less gonadotoxic, such as ABVD, could be used as a fertility preservation strategy (discussed below and in Chapter 3) (Viviani *et al.*, 1985). Cancer treatment can have a direct impact on the endocrine system damaging the hypothalamus and/or anterior pituitary within the HPG axis, which would lead to the observations in the previously noted studies. Analysis of the direct damage will therefore provide clearer information on chemotherapy-induced damage.

1.4.3 Summary of Evidence from Human Studies

The studies discussed above suggest that alkylating agents, in particular cyclophosphamide, can be detrimental to the prepubertal testis in a manner that can persist at least up to ten years after the cessation of treatment. Analysis of hormonal changes and semen parameters could provide an indirect measurement of gonadal damage; however, such data may not indicate direct damage to the gonadal system,

rather damage to the HPG axis. Since patients receive a combination of chemotherapy drugs for different lengths of time, it is hard to determine the relative contributions of individual chemotherapeutic drugs to gonadal toxicity; in addition, results may have been influenced by the age/stage of pubertal development and each patient's own sensitivity to chemotherapy treatments accounting for differing observations from the above studies.

1.5 Evidence of Chemotherapy-induced Damage to Prepubertal Testis from Animal Model Studies

The use of animal models has the potential to provide a clearer picture of chemotherapy drug-induced gonadal toxicity in the prepubertal context, in comparison to our very limited ability to investigate this directly in humans. Research on animal models enables researchers to administer drugs through more regulated regimens and to compare results using animals, tissues or cells. The use of animal models also opens up the possibility of determining which period of development is more sensitive to chemotherapy treatment, for example by comparing infancy to prepuberty. Additionally, such studies are likely to be invaluable in determining the underlying mechanisms by which the different chemotherapy drugs damage the prepubertal testis, information which should help in the subsequent development of protective strategies designed to directly block such damage. Due the scarcity of human prepubertal testis tissue, potential species differences need to be considered when interpreting these results. Despite their great potential, relatively few studies have been conducted *in vivo* or *in vitro*, with the majority of studies focusing on germ cell effects. Work to date has looked specifically at alkylating agents, anthracyclines, topoisomerase inhibitors, vinca alkaloids and antitumour antibiotic chemotherapy treatments.

1.5.1 Alkylating & Alkylating-Like Agents

As in human studies, research to date using prepubertal testes of rodents points to alkylating and alkylating-like agents targeting the germ cell population in a dose- and

time- dependent manner (Table 1.3A). To date, investigations have shown that SSCs are sensitive to cyclophosphamide and cisplatin, with treatment inducing DNA damage *in vitro* which could ultimately activate cell death pathways if not repaired by internal DNA repair systems (Marcon *et al.*, 2010; Liu *et al.*, 2014). DNA damage was noted following short-term (24 hours (hrs)) exposure to chemotherapeutics using an *in vitro* culture system of prepubertal (pnd five) mouse testis (Smart *et al.*, 2018). Activation of the apoptotic pathway was shown by enhanced cleaved caspase (CC) 3 expression following cyclophosphamide and cisplatin treatment with the timings differing between the agents, followed by loss of germ cells hrs later. Increased numbers of apoptotic spermatogonia and primary spermatocytes have also been observed *in vivo* immediately following cisplatin treatment during early puberty (pnd 30-45) in the rat and after a 45-day recovery period (Lirdi *et al.*, 2008; Favareto *et al.*, 2011). Survival of spermatogonial cells and somatic cells are crucial for restoration of spermatogenesis following treatment. A study by Abumadighem *et al.* (2018) treated prepubertal mice (pnd 7) *in vivo* with a toxic dose of busulfan (45mg/kg), after which isolated testicular cells were cultured in a 3D *in vitro* culture system for up to four weeks. Meiotic and post-meiotic germ cells formed in culture, with formation of sperm-like cells following the addition of testis homogenate from GFP-expressing adult mice. Formation of sperm-like cells did not occur with incubation of immature testis homogenate, indicating that growth factors and signalling molecules in the adult testis are required. This research shows that even with exposure to high doses of alkylating agents, survival of testicular cells and recovery of spermatogenesis are still possible. The length of the treatment rather than the cumulative doses appears to be involved in determining the degree of testicular damage (Velez de la Calle *et al.*, 1989). Shorter treatment regimens reduce exposure of the testis to the chemotherapeutic agent, which could be more important than overall cumulative dose received.

Table 1.3: Animal model data studying gonadotoxicity of alkylating and alkylating-like chemotherapy agents. Updated since publication in Allen *et al.* (2018)

Chemotherapy Drug	Doses (Length of Treatment)	Animal Model & Age	*In vivo* or *In vitro*	Effect		Papers
				Germ Cells	Somatic Cells	
Busulfan	45mg/kg (single injection)	Prepubertal mice (pnd 7)	*In vivo* exposure & *in vitro*	↑damage seminiferous tubules with recovery noted 6wks after treatment ↓Pre-meiotic GCs (VASA & SALL4), meiotic GCs (CREM-1) & meiotic and post-meiotic GCs (ACROSIN) ↓ proliferation Were capable of producing post-meiotic cells 4wks in culture		Abumadighem *et al.*, 2018
Cyclophosphamide (phosphoramide mustard metabolite)	0.02, 0.2 and 2µg/ml (24 hrs)	Prepubertal mouse (pnd5)	*In vitro*	↓GCs, particularly SSCs ↑CC3 expression 16 hrs after treatment, ↓GCs eight hrs later. ↑γH2AX GC expression 16 hrs after treatment.	No effect on SC or LC numbers.	Smart *et al.*, 2018
Cyclophosphamide	0.04-0.28g/kg (2 wks)	Prepubertal rat (pnd10)	*In vivo*	No evidence of testicular damage.	N/A	Velez de la Calle *et al.* 1989
	0.002g/kg/day (5 wks) 0.7g/kg total	Pubertal rat (pnd45)	*In vivo*	Atrophied seminiferous tubules ↓spermatogonia and primary spermatocytes.	Vacuolation of SC. ↓ ABP production.	

	0.1g/kg (10 days)	Pubertal rat (pnd45)	*In vivo*	No evidence of testicular damage.	N/A	
Procarbazine	0.030g/kg/day (5 or 9 wks)	Prepubertal (pnd10), pubertal (pnd45) & adult rat (pnd70-90)	*In vivo*	5 wks; ↓diameter of SCO (prepubertal), less of an effect on pubertal and adults 9 wks; less impact on pubertal rats.	Vacuolation of SC. ↓ ABP production.	Velez de la Calle *et al.* 1988
Cyclophosphamide (4-hydroperoxy cyclophosphamide -4OOH-CPA metabolite)	0.1µM (48 hrs)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑ γH2AX telomeres expression. ↓telomere length and activity reduced.	N/A	Liu *et al.* 2014
Cyclophosphamide (arcolein metabolite)	50 or 100µM (3 or 12 hrs)	Mouse SCs (isolated from pnd8)	*In vitro*	N/A	↓ viability of SC. ↑ ROS and ↓ antioxidant activity ↑ apoptosis. Impairs cytoskeleton of SCs.	Liu *et al.*, 2012
Cisplatin	10-15µg/ml	Mouse SSCs (isolated from pnd3-6)	*In vitro*	↓survival of SSC clusters in dose-dependent manner.	N/A	Shabani *et al.* 2016
	0.1, 0.5 and 1µg/ml (24 hrs)	Prepubertal mouse (pnd5)	*In vitro*	↓GCs, particularly SSCs ↑CC3 expression 24 hrs after treatment, ↓GCs eight hrs later.	No significant effect on SC or LC numbers.	Smart *et al.* 2018

				↑γH2AX GC expression 16 hrs after treatment.		
	1µM (48 hrs)	Rat SSCs (isolated from pnd7-8)	*In vitro*	Dose-dependent ↑γH2AX.	N/A	Marcon *et al.*, 2010
	0.5µg/ml (24 hrs)	SCs prepubertal rat (pnd18)	*In vitro*	N/A	50%↓ transferrin production.	Nambu *et al.*, 1995
	200 or 400µM (24 hrs)	SCs prepubertal rat (pnd19)	*In vitro*	N/A	↓ cell viability (200µM) but no ↑CC3 activation at 24 hrs.	Aslani *et al.*, 2017
	0.005g/kg	Pubertal rat (pnd30)	*In vivo*	↓seminiferous epithelium. ↑apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes 12 hrs after treatment.	N/A	Lirdi *et al.*, 2008
	0.001g/kg/day (3 wks)	Pubertal rat (pnd45)	*In vivo*	↑apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes.	Vacuolation of SC. ↓ ABP production.	Favareto *et al.*, 2011
	0.1µM (48 hrs)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑γH2AX telomeres. ↓telomere length and activity of telomerase	N/A	Liu *et al.*, 2014

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; peritubular myoid cells, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.

1.5.2 Anthracyclines

Of the anthracycline class of drugs, work to date has focused on doxorubicin, and it is clear this drug targets the pre-mitotic dividing spermatogonia and the pre-meiotic primary spermatocytes in the prepubertal testis (Table 1.4). These germ cells are undergoing DNA synthesis and therefore contain high levels of the enzyme topoisomerase II which is a target of the drug (Parvinen and Parvinen, 1978). Indeed, doxorubicin treatment of the GC-1 cell line, representative of type B spermatogonia and preleptotene spermatocytes, resulted in increased numbers of these cells arrested at the G2/M phase checkpoint of the cell cycle (Akinjo *et al.*, 2018). This checkpoint is the DNA damage checkpoint assessing for errors during DNA synthesis. During early prepubertal development, the testis is especially vulnerable to doxorubicin-induced damage, depleting the seminiferous epithelium in comparison to later stages shown in the rat model (Bechter *et al.*, 1987). This study found using an immature *in vivo* rat model, that a relatively low dose of doxorubicin (3mg/kg) was not sufficient to kill all the SSCs, as some recovery was apparent. In addition to inhibition of topoisomerase II activity, doxorubicin has been shown to induce DNA damage in spermatogonia *in vitro*, which can result in cell death (Beaud *et al.*, 2017). Interestingly, the work of Beaud *et al.* (2017) indicated that cell death was induced independent of apoptosis, since externalization of phosphatidylserine was not apparent following treatment. In agreement with these findings, DNA damage was observed despite the absence of a significant increase in CC3 expression prior to loss of germ cells following short-term exposure of prepubertal mouse testis tissue to doxorubicin (Smart *et al.*, 2018). The cell death that occurs following doxorubicin may result from a non-apoptotic mechanism for example through necrosis or autophagy (Beaud *et al.*, 2017; Smart *et al.*, 2018).This hypothesis would be consistent with studies analysing cardiotoxicity following doxorubicin treatment where the cell death induced is through autophagy (reviewed in Dirks-Naylor, 2013). However, increased levels of CC8 and p53 48 hrs after treatment in an *in vivo* model has also been reported (Hou *et al.*, 2005). Oxidative stress has also been proposed as a mechanism of doxorubicin-induced damage to germ cells, however work in an *in vitro* model

culturing a cell line with rat SSC/spermatogonia type A characteristics (GC-6spg) has shown no such increase in levels of reactive oxygen species (ROS) before the onset of cytotoxicity (Tremblay and Delbes, 2018).

Table 1.4 Animal model data studying gonadotoxicity of anthracyclines. Updated since published in Allen *et al.* (2018)

Doses of Doxorubicin & Length of Treatment	Animal Model & Age	*In vivo* or *In vitro*	Effect		Papers
			Germ Cells	Somatic Cells	
40 and 100ng/ml (up to 72 hrs)	Prepubertal rat (pnd5)	*In vitro*	↓GC numbers 24 and 48 hrs, ↓proliferating and ↑apoptotic GCs, after 16 hrs.	No effect SCs number, production of key proteins. No impact on PMC proliferation or LC testosterone production.	Nurmio *et al.*, 2009b
0.05, 0.1 and 0.5µg/ml (24 hrs)	Prepubertal mouse (pnd5)	*In vitro*	↓GCs, particularly SSCs. No expression of CC3 observed before GC loss. ↑γH2AX GC expression 16 hrs after treatment.	No effect on SC or LC numbers.	Smart *et al.*, 2018
0.003g/kg	Prepubertal (pnd6 & 16) & pubertal rat (pnd24 & 45)	*In vivo*	Prepubertal rats ↑SSC death. Toxicity reduced in pubertal rats 3, 7 and 14 days after treatment.	N/A	Bechter *et al.*, 1987
0.003g/kg	Prepubertal rat (pnd6, 16 and 24)	*In vivo*	Targets migrating gonocytes. ↑apoptotic cells in pnd6 rats 48 hrs after treatment, ↑ p53 & CC8 expression.	N/A	Hou *et al.*, 2005
0.4µg/ml (24 hrs)	SCs prepubertal rat (pnd18)	*In vitro*	N/A	35% ↓transferrin production.	Nambu *et al.*, 1995

0.005g/kg	Pubertal rat (pnd22)	*In vivo*	N/A	Dysfunction and morphological alterations pnd40, more advanced pnd64 with recovery pnd127 (adult). ↓ transferrin production and abnormal positioning of SC nuclei.	Brilhante *et al.*, 2012
0.1µM (24-72 hrs)	GC-6 spermatogonial cell line (rat)	*In vitro*	DNA strand breaks. Cell death without activation of apoptosis (externalization of phosphatidylserine).	N/A	Beaud et al., 2017
0.01-10µM (24 hrs)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Time- and dose-dependent ↑ in cytotoxicity.	Time- and dose-dependent ↑ in cytotoxicity (Ser-W3 more sensitive). ↑Oxidative stress, nuclear 8-oxo-deooxyguanosine ↓glutathione levels 6 hrs. Glutathione supplementation did not affect survival.	Tremblay and Delbes, 2018
1nM - 5µM (24 – 48hrs)	GC-1 spermatogonial TM3 Leydig cell TM4 Sertoli cell	*In vitro*	GC-1 most sensitive to cell-cycle arrest at G2/M phase and ↓ in % S-phase cells Dose- and time-dependent differential expression of miRNA (101)	TM4 and TM3 cell-cycle arrest at G2/M phase & ↓ in % S-phase cells, TM4 most resistant Dose- and time-dependent differential expression of miRNA (TM4; 117, TM3; 64)	Akinjo *et al.*, 2018

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; PMCs, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.

1.5.3 Topoisomerase Inhibitors

Topoisomerase inhibitors, such as etoposide and irinotecan, have been investigated in order to determine prepubertal gonadal toxicity and have been found to target the pre-mitotic and pre-meiotic germ cells (Table 1.5). These drugs inhibit the activity of the enzyme topoisomerase I and II and ultimately induce cell death through activation of apoptotic pathways (Freitas *et al.*, 2002; Stumpp *et al.*, 2004; Ortiz *et al.*, 2009). Etoposide damages the prepubertal testis depleting the germ cell pool, with little recovery from treatment at low doses (2mg/kg; Freitas *et al.*, 2002). SSCs are particularly vulnerable to etoposide treatment, with a lower half maximal inhibitory dose in comparison to cisplatin and bleomycin. With etoposide targeting the SSC population, this reduction could account for the reduction in later stages of spermatogenesis observed in the adult rat (Stumpp *et al.*, 2004; Marcon *et al.*, 2010). Exposure of the prepubertal mouse testis *in vitro* to concentrations of SN38 (the metabolite of irinotecan) that reflect patient serum levels shows that this drug targets the proliferating germ cell population (Lopes *et al.*, 2016). Involvement of the apoptotic pathway in cell death has been observed following etoposide treatment in an *in vivo* prepubertal rat model, with increased numbers of apoptotic intermediary and type B spermatogonia, as well as primary spermatocytes, immediately following treatment (Stumpp *et al.*, 2004). In addition, increased activity of CC9, CC3 and CC8 as well as enhanced levels of protein and mRNA of p53 and Bcl-2 have been observed in the prepubertal rat following etoposide treatment (Ortiz *et al.*, 2009). However, from this study it is not possible to determine which cell types within the testis the observed changes occurred in, since results were obtained from homogenised tissue.

Table 1.5: Animal model data studying gonadotoxicity of Topoisomerase Inhibitors. As published in Allen *et al.*, (2018)

Chemotherapy Drug	Doses & Length of Treatment	Animal Model & Age	*In vivo* or *In vitro*	Effect		Papers
				Germ Cells	Somatic Cells	
Etoposide	1µM (48 hrs)	Rat SSCs (isolated from pnd7-8)	*In vitro*	↓SSC clusters.	N/A	Marcon *et al.*, 2010
	25µM or 100µM (24 hrs)	SCs from prepubertal rat (pnd19)	*In vitro*	N/A	No impact on cell viability or CC3 activation.	Aslani *et al.*, 2017
	1.2µg	Prepubertal rat (pnd21)	*In vivo*	↑CC9, 8 and 3 activation 24 hrs after treatment in spermatocytes. Protein and mRNA of p53 and Bcl2 altered.	N/A	Ortiz *et al.*, 2009
	0.05g/kg/day Total doses 0.01, 0.02 & 0.04g/kg	Prepubertal rat (pnd25)	*In vivo*	Analysed pnd26 and pnd32. ↑apoptotic differentiated spermatogonia and primary spermatocytes except for 0.01g/kg group when sacrificed 12 hrs after end of treatment.	N/A	Stumpp *et al.*, 2004
	0.04g/kg	Prepubertal rat (pnd25)	*In vivo*	N/A	SC adluminal with chromatin clumps and vacuolization. ↓transferrin production from pnd45 onwards.	Stumpp *et al.*, 2006, 2008

	0.002g/kg (30 days)	Pubertal rat (pnd30)	*In vivo*	↓GCs, ↑ cell death (chromatin condensation). Damage still apparent 113 days after treatment.	N/A	Freitas *et al.*, 2002
	0.01µM (48 hrs)	C18-4 spermatogonial cells (mouse)	*In vitro*	No effect on levels of γH2AX levels in telomeres or telomere dysfunction.	N/A	Liu *et al.*, 2014
Irinotecan (SN38 metabolite)	0.1 and 1µg/ml (24 hrs)	Prepubertal rat (pnd5)	*In vitro*	Targets the proliferating germ cell population.	N/A	Lopes *et al.*, 2016

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; PMCs, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.

1.5.4 Vinca Alkaloids & Antitumor Antibiotics

In vitro studies indicate that vincristine may target the germ cell population and bleomycin has the potential to damage the testis (Table 1.6). Vincristine reduces cell viability and increases cell death of the GC-6spg cell line in a dose- and time-dependent manner. However this was not a result of DNA damage, as this class of drugs inhibits polymerization of microtubules, involving activation of apoptotic pathways (Beaud *et al.*, 2017). Bleomycin targets SSCs *in vitro* in a dose-dependent fashion, with cytotoxicity (seen at 0.1µM) inducing DNA damage that extends into telomere regions of chromosomes (Marcon *et al.*, 2010; Liu *et al.*, 2014). These initial studies indicate that such drugs have the potential to impact the testis negatively and should be further investigated *in vivo*. Vincristine is investigated in this thesis to determine gonadotoxic insult to the prepubertal testis with cultured prepubertal mouse testis fragments and a cell line representative of SSCs, the GC-6spg cell line (see Chapter 4).

Table 1.6 **Animal model studies investigating gonadotoxicity of vinca alkaloids or antibiotic agents.** As published in Allen *et al.*, (2018)

Chemotherapy Drug	**Doses & Length of Treatment**	**Animal Model & Age**	***In vivo* or *In vitro***	**Effect**		**Papers**
				Germ Cells	Somatic Cells	
Vincristine	0.1µM (48 hrs)	Rat SSCs (isolated from pnd7-8)	*In vitro*	Targets SSC in a dose-dependent manner.	N/A	Marcon *et al.*, 2010
	0.5µg/ml (24 hrs)	SCs from prepubertal rat (pnd18)	*In vitro*	N/A	↓transferrin production.	Nambu *et al.*, 1995
	0.1µM (24-72 hrs)	GC-6 spermatogonial cell line (rat)	*In vitro*	↓cell viability, ↑cell death differentiated spermatogonia dose- and time-dependent manner. No observable DNA damage but activation of apoptotic pathways.	N/A	Beaud *et al.*, 2017
Bleomycin	0.1µM (48 hrs)	Rat SSCs (isolated from pnd7-8)	*In vitro*	↓cluster number and area in culture.	N/A	Marcon *et al.*, 2010
	1.5µM (48 hrs)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑DNA damage in the telomeres, no impact on telomerase activity.	N/A	Liu *et al.*, 2014
Mitomycin C	500µg/kg (alternate day for 20 dys)	Pubertal rats (pnd 40)	*In vivo*	N/A	↓Leydig cell nuclear area ↓ 3β-Hydroxysteroid dehydrogenase	Deb *et al.*, 1980

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; peritubular myoid cells, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.

1.5.5 Combination Treatments

In a clinical setting, cancer is usually treated with a combination of chemotherapeutic agents. Different classes of drugs target cells through different mechanisms, which may have synergistic effects. The combined treatments reduce the chance of resistance and survival of cancerous cells, but have the possibility of leading to multiplicative off-target side effects on healthy cells. Relatively few animal studies have focused on combinations of drugs (Table 1.7). Using the GC-6spg cell line, Beaud *et al.* (2017) treated cells with a combination of vincristine and doxorubicin that enhanced levels of cell death in a dose-dependent manner in comparison to treatment with each drug individually. In contrast, exposure to a combination of bleomycin, cisplatin and etoposide had no additional impact on cluster size/area of mouse SSCs in culture (Marcon *et al.*, 2010). Further investigations into common combinations are required to determine relative gonadotoxicity, and to investigate whether effects are synergistic and/or multiplicative.

Table 1.7 Animal model studies investigating gonadotoxicity of combination chemotherapy treatments. As published in Allen *et al.*, (2018)

Chemotherapy Drug	**Doses & Length of Treatment**	**Animal Model & Age**	***In vivo* or *In vitro***	**Effect**		**Papers**
				Germ Cells	Somatic Cells	
Bleomycin, Cisplatin & Etoposide	0.1µM (48 hrs)	Rat SSCs (isolated from pnd7-8)	*In vitro*	Combination had no additional impact on cluster size/area of SSCs.	N/A	Marcon *et al.*, 2010
Vincristine & Doxorubicin	0.01µM (24-72 hrs)	GC-6 spermatogonial cell line (rat)	*In vitro*	Combination ↑cell death of spermatogonia dose-dependent manner in comparison to individual treatment.	N/A	Beaud *et al.*, 2017

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; PMCs, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.

1.5.6 Somatic Cell Effects

Studies relating to impacts of chemotherapy treatment upon the somatic cells has mainly been limited to the Sertoli cell and includes studies which have focused on cyclophosphamide, cisplatin, doxorubicin and etoposide chemotherapy agents (see above). Treatment results in morphological damage including vacuolation of the cells and adluminal positioning (Velez de la Calle *et al.*, 1988, 1989, Stumpp *et al.*, 2006, 2008; Favareto *et al.*, 2011; Brilhante *et al.*, 2012). However, these cells do survive exposure as shown in Smart *et al.* (2018), where there was no overall change in cell numbers following cyclophosphamide, cisplatin or doxorubicin treatment in an *in vitro* model of prepubertal mouse testicular tissue. Damage to the Sertoli cells resulting in dysfunction could be the primary effect of such treatment and would have a significant impact on testis function. Indeed, decreased production of androgen binding protein following treatment with alkylating and alkylating-like agents has been reported in prepubertal rats (Velez de la Calle *et al.*, 1988, 1989; Favareto *et al.*, 2011). *In vitro* studies using primary cultures of rat Sertoli cells have also shown reduced transferrin production following cisplatin, doxorubicin and vincristine treatment (Nambu *et al.*, 1995). Transferrin stimulates germ cell proliferation/differentiation by transferring iron to these cells (reviewed in Sylvester and Griswold, 1994). A potential mechanism by which cyclophosphamide and doxorubicin induce damage specifically to the Sertoli cells has been proposed by *in vitro* studies of cultured immature Sertoli cells where enhanced levels of oxidative stress have been reported (Liu *et al.*, 2012; Tremblay and Delbes, 2018) as well as damage to the cytoskeleton following cyclophosphamide treatment (Liu *et al.*, 2012).

Impairment of Sertoli cell functionality, however, may be secondary and a result of primary injury to the germ cell population. An *in vivo* study investigating doxorubicin-induced damage has shown that the alterations in the morphology and function of these cells was more pronounced in early adulthood in comparison to time of treatment just before puberty (pnd 22). This suggests that the germ cells were the primary cells targeted by doxorubicin, with loss/damage to the germ cells ultimately

impacting Sertoli cells as a secondary effect (Brilhante *et al.*, 2012). For etoposide, functional deficits were also more apparent in adulthood, suggesting damage was secondary to germ cell death. However, upon recovery of the seminiferous epithelium, Sertoli cell dysfunction was still apparent with reduced transferrin production and altered morphology; this may suggest a degree of primary damage on the Sertoli cells (Stumpp *et al.*, 2006). How to distinguish between primary and secondary damage to the Sertoli cells is difficult due to the dependence of Sertoli cells on germ cells and vice versa. Isolation of Sertoli cells from the germ cell population, as with the primary cell culture or established cell line culture experiments, has been used to look specifically at Sertoli cell damage. These isolated Sertoli cells, however, may not be representative of the 'true' *in vivo* Sertoli cell as often these cells do not maintain Sertoli cell identity once removed from their true environment, a limitation that needs to be borne in mind for all cell line studies (see Chapter 7.3.2). However, research with the TM4 Sertoli cell line, TM3 Leydig cell line and GC-1 cell line (derived from immature mice) has shown differential miRNA expression following doxorubicin treatment. These include miRNA and mRNA targets that are associated with germ cell-Sertoli cell communication and Sertoli cell-Sertoli cell communication, in addition to reproductive diseases and developmental disorders (Akinjo *et al.*, 2018). This research highlights the importance of cell-to-cell communication within the testis that could be disrupted following chemotherapy treatment.

To date, few additional studies have focused on the other somatic cell types within testis, the Leydig cells or PMCs. An *in vivo* study reported reduced steroidogenic activity of Leydig cells following treatment of immature rats with mitomycin C (Deb et al., 1980). More recent *in vitro* studies have shown there was no change in Leydig cells numbers reported by Smart *et al.* (2018) after *in vitro* exposure of mouse prepubertal testis fragments to cisplatin, cyclophosphamide or doxorubicin. The proliferative ability of the PMCs and steroidogenic activity of the Leydig cells were also unaltered after doxorubicin treatment of rat testis tissue *in vitro* (Nurmio *et al.*,

2009b). Additional studies looking specifically at these cell types within the testis are required before any conclusion can be drawn on chemotherapy-induced damage to these cell types.

1.5.7 Summary of Evidence derived from Animal Studies

To conclude, in the prepubertal testes chemotherapy agents have been shown to specifically target and deplete the germ cell pool, in some cases specifically the SSC population, with DNA damage noted after cyclophosphamide, cisplatin and doxorubicin exposure. Apoptosis is the main cell death pathway activated by cyclophosphamide, cisplatin, etoposide and vincristine exposure, whereas doxorubicin-induced testicular damage may be the result of an alternative cell death pathway such as necrosis or autophagy. Cancer therapy may also affect the Sertoli cell population resulting in morphological damage and/or dysfunction, as shown by cyclophosphamide, procarbazine, cisplatin, doxorubicin and etoposide treatment. However, somatic cell impairment may either be the result of primary cellular damage to the Sertoli cells themselves and/or a secondary consequence of targeted loss of the germ cell population. Whether combined chemotherapy treatment regimens modify the toxicity of individual drugs needs further clarification as few such *in vitro* studies have been performed to date. In this thesis, the role of oxidative stress in cisplatin-, doxorubicin- or vincristine-induced damage to the prepubertal mouse testis and rat GC-6spg cell, representative of SSCs, will be further explored (Chapter 5).

1.6 Effects on Future Generations

The clinical impact of prepubertal chemotherapy treatment on later fertility has been discussed in several reviews (Hudson, 2010; Lee and Shin, 2013; Delessard *et al.*, 2020); for survivors who are able to conceive there could be potential effects upon future generations due to unrepaired damage to the male germline. Studies evaluating semen quality following chemotherapy treatment during childhood found that even though men had reduced sperm concentrations, the quality of the sperm

in relation to DNA and chromatin integrity were normal compared to non-treated men (Thomson *et al.*, 2002; Romerius *et al.*, 2010). The impact on future generations is not yet clear, men who have survived childhood cancer are less likely to father children, of which there are multiple confounding factors excluding infertility (Tang *et al.*, 2016). Studies have increasingly found that children of men who survived childhood cancer are at no increased risk of genetic disease or mortality compared to control populations or their siblings (Meistrich and Byrne, 2002; Signorello *et al.*, 2012; Winther *et al.*, 2012; Seppänen *et al.*, 2016; Tang *et al.*, 2016). Recent research, has however, indicated the potential for epigenetic changes to the male germ line where differential DNA methylation sites within the DNA of the sperm of survivors of childhood cancers were found, which may have an impact on future progeny but has yet to be identified by current studies (Shnorhavorian *et al.*, 2017). A study by Liu *et al.* (2014) has shown a potential mechanism by which alkylating agents can impact on future generations by targeting the telomeres of mouse spermatogonial cells. Telomerase function was reduced at concentrations of a precursor of cyclophosphamide (4OOH-CPA) or cisplatin, which induced significant spermatogonial cell death resulting in reduced telomere length and telomerase activity. A reduction in the length or function of telomeres of the male germ cell can adversely affect early development of the offspring, increasing the rate of pre- and/or post-implantation loss, congenital malformation and miscarriage (Liu *et al.*, 2002). Whether additional classes of drugs also impact on future generations is unknown and therefore further research into this area is urgently required.

1.7 Fertility Protective Strategies

The development of chemotherapeutic treatment regimens has increased greatly in the past decade, resulting in a greater number of childhood cancer survivors reaching adulthood and facing long-term consequences of treatment such as infertility. There is therefore an increasing focus on preserving the fertility of children undergoing cancer treatment (Fig 1.3) (reviewed in Tournaye *et al.*, 2014; Picton *et al.*, 2015; Goossens *et al.*, 2020). At the time of writing the only options to preserve fertility are

testicular shielding if undergoing radiotherapy and use of chemotherapy agents that are considered 'less gonadotoxic', with the latter relying on a classification system that is often debated (Viviani *et al.*, 1985; reviewed in Wallace and Thomson, 2003; Allen *et al.*, 2018). Effective fertility preservation strategies for prepubertal boys need to be established clinically (Fig 1.3). Testicular tissue from boys undergoing 'high' risk chemotherapy treatment is being collected in a limited number of centres, with tissue cryopreserved and stored for potential fertility restoration when the boys reach adulthood. The proposed techniques to restore fertility, however, are still in the experimental phase of development, with success to date only in animal models (reviewed in Picton *et al.*, 2015; Giudice *et al.*, 2017; Goossens *et al.*, 2020). Studies with human tissues have been conducted in relation to transplantation of cryopreserved testicular tissue or SSCs, as well as *in vitro* maturation of immature testicular tissue, paving the way for the development of a clinical fertility restoration method (reviewed in Picton *et al.*, 2015; Giudice *et al.*, 2017; Goossens *et al.*, 2020).

An alternative strategy that could be used to protect the prepubertal testis against injury caused by the treatment, or to stimulate recover following treatment, includes the use of cytoprotective agents that would be added before, during or after cancer treatment. So far, only a limited number of compounds have been analysed to determine their ability to protect the prepubertal testis in animal models. For all such potential fertility preservation techniques, further research is needed to allow the methods to be successfully transformed for use with human tissue, after which further validation will be required to ensure that such methodologies are efficient and safe for clinical use.

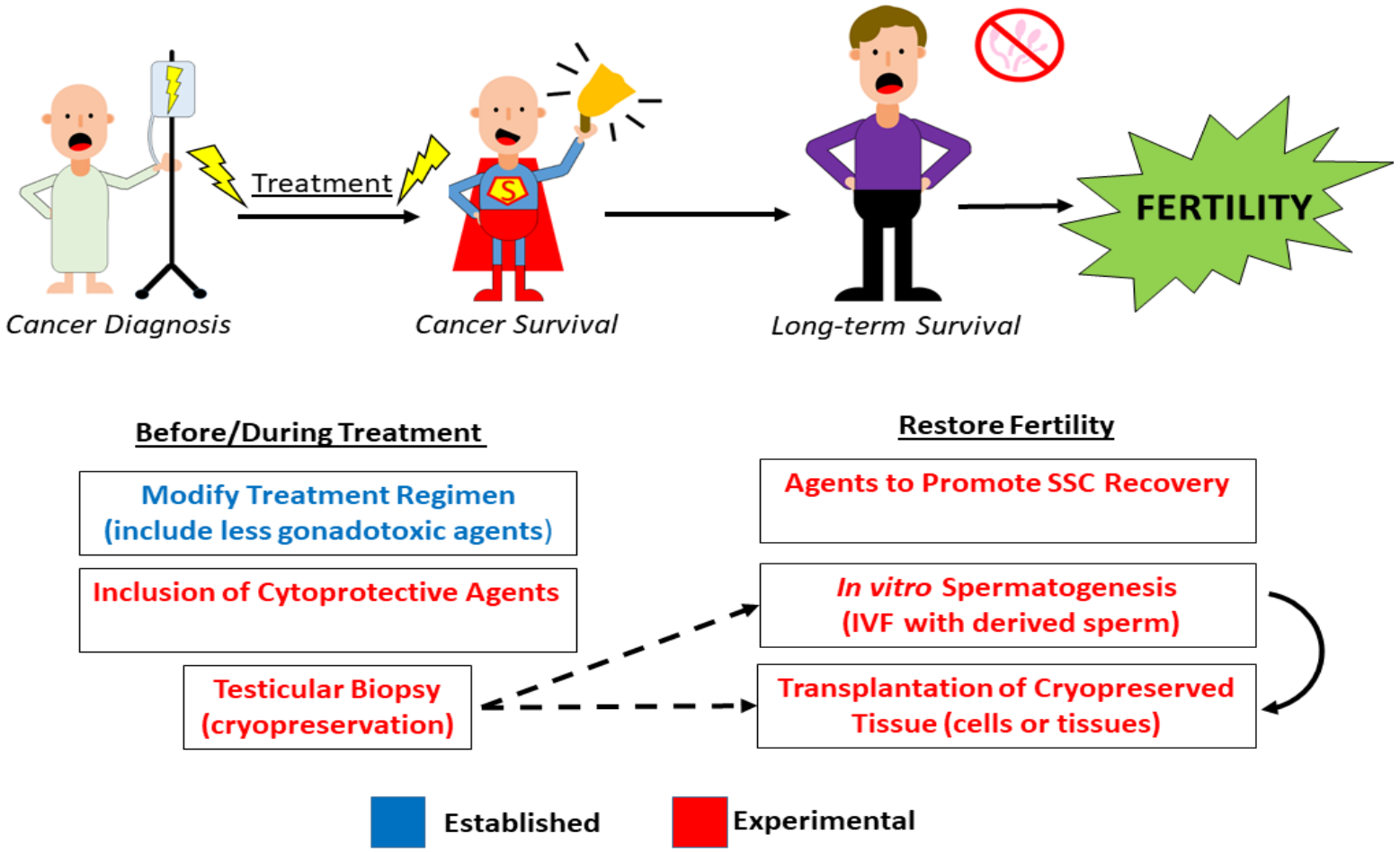


Figure 1.3: Current (blue) and experimental (red) methods of fertility preservation for young boys diagnosed with cancer. Modified from Kilcoyne and Mitchell, (2019a).

1.7.1 Treatment of Infertility

Research into the restoration of fertility in adulthood following childhood cancer treatment has investigated the potential of auto-transplantation of cryopreserved prepubertal testis fragments or *in vitro* spermatogenesis. However, these techniques have only been successful in restoring fertility in animal models. Research is further along for females; auto-transplantation of ovarian tissue cryopreserved from the post-pubertal ovary has resulted in 100s of live births (reviewed in Anderson and Baird, 2019). Indeed, there are have now been two live births from auto-transplantation of ovarian tissue cryopreserved from young girls; one from a prepubertal girl aged nine and another from a 13 year old girl who had entered puberty based on breast development but had yet to menstruate (Demeestere *et al.*, 2015; Matthews *et al.*, 2018). This technique is still 'experimental', but hopefully will in the future become clinically established for young girls undergoing cancer treatment. More work is required however, to restore later fertility from testis tissue cryopreserved before puberty to preserve fertility for young boys.

1.7.1.1 Testicular Biopsy and Cryopreservation

Research centres have recently begun to cryopreserve immature testicular tissue from prepubertal boys before the commencement of chemotherapy treatment or for other medical conditions such as Klinefelter syndrome or Fanconi syndrome. In 2019, there were 24 sites collecting such tissues in Europe, with around 1033 testis samples stored (reviewed in Goossens *et al.*, 2020). Importantly, biopsies are only undertaken when treatment is deemed at high risk for later fertility complications (reviewed in Picton *et al.*, 2015; Mitchell *et al.*, 2017; Goossens *et al.*, 2020), with no long-term consequences on testis growth noted after biopsy (Uijldert *et al.*, 2017). At this point, however, it is not yet certain if such cryopreserved tissue can be successfully used later to restore fertility in humans, since production of viable sperm from such tissue has yet to be achieved. Optimization is also still required for patient selection and cryopreservation techniques. In regards to patient selection, guidelines have been published, however, there are no clinical recommendations published with different

centres using different selection criteria (reviewed in Anderson *et al.*, 2015). A standard cryopreservation protocol has yet to be established with optimization required for overall methodology (controlled/uncontrolled slow freezing or vitrification), cryoprotectant used (DMSO or glycerol based), whether to cryoprotect whole tissue fragments or cell suspensions and thawing protocols (reviewed in Kilcoyne and Mitchell, 2019a). A review of the literature by Zarandi *et al.* (2018) evaluated current protocols and came to the conclusion that slow freezing methodology with DMSO has more promise as a standardised approach compared to vitrification, which is a more complicated procedure requiring rapid ultra-cooling of tissue to prevent ice crystal formation (Keros *et al.*, 2007; Onofre *et al.*, 2016). Survival of SSCs have, however, been reported with both slow-freezing and vitrification techniques when cryopreserving human immature testicular tissue (Poels *et al.*, 2013).

1.7.1.2 Transplantation of Testicular Tissue

Research in animal models has been more successful in showing the potential of transplantation of testicular tissue/SSCs as a fertility preservation strategy which could be developed for clinical use (reviewed in Picton *et al.*, 2015; Giudice *et al.*, 2017). Xenotransplantation of frozen-thawed immature testicular tissue and transplantation of SSCs has been successful, with completion of spermatogenesis found in animal models including the non-human primate and rodents (Brinster and Zimmermann, 1994; Luetjens *et al.*, 2008; Hermann *et al.*, 2012; Jahnukainen *et al.*, 2012; Ntemou *et al.*, 2019a). Healthy offspring have been produced through IVF/ICSI using sperm derived from xenografted non-human primate immature testicular tissue demonstrating the potential of this technique for clinical application (Liu *et al.*, 2016). Xenotransplantation of prepubertal human testicular tissue for up to 9 months has been performed with nude mice where initiation of spermatogenesis was observed within the grafted tissue (Wyns *et al.*, 2007, 2008; Goossens *et al.*, 2008; Sato *et al.*, 2010; Van Saen *et al.*, 2011, 2013; Poels *et al.*, 2013). Spermatogenesis, however, did not reach meiotic differentiation in a subset of these studies with few

reporting presence of spermatocytes (Wyns *et al.*, 2008; Sato *et al.*, 2010; Poels *et al.*, 2013; Van Saen *et al.*, 2013). Xenotransplantation, however, may not be the most appropriate methodology for fertility preservation due to possible transmission of zoonosis from the host animal (reviewed in Mitchell *et al.*, 2009). In the rhesus monkey, recent success has shown the potential of autologous transplantation of immature, cryopreserved prepubertal testis back in to the adult, where sperm derived from scrotal grafts were viable, capable of fertilizing an oocyte and resulted in a live birth (Fayomi *et al.*, 2019). This study was a major step towards developing a clinical methodology of autologous transplantation, comparable to that currently performed for females. There are concerns, however, for non-solid tumours that malignant cells could be reintroduced back into the patient, particularly with tissue transplantation, as shown in the Hou *et al.*, (2007) study where leukemic infiltration was noted within the xenografted testicular tissue (reviewed in Kilcoyne and Mitchell, 2019a). However, there is also evidence from a prepubertal primate model of the possibility to sorting out cancer cells from amongst SSCs based on fluorescent activated cell sorting (Hermann *et al.*, 2011).

1.7.1.3 *In Vitro* Spermatogenesis

The production of viable sperm *in vitro* has been successful in mouse models where sperm produced were used for IVF/ICSI to produce healthy offspring (Sato *et al.*, 2011b, a). This technique would reduce the potential of reintroducing malignant cells back into the body and potentially increase the number of germ cells for transplantation where smaller volumes of testis biopsy, and therefore germ cells, are collected. Recent reports of complete spermatogenesis following culture of human fetal testicular tissue (weeks 12 to 19) for around 50 days has been described in Yuan *et al.* (2020). Of 44 'gonadal samples' that were cultured, 23 derived from three fetuses, produced haploid sperm-like cells. The remaining samples, however, were reported to have undergone apoptosis due to low sample quality and it is unknown if these sample were derived from the same above fetuses. The functional capacity of 16 of these cells was assessed by round spermatid injection of healthy oocytes and

subsequent embryo culture up to the eight-cell stage, with fertilization rates and developmental competency similar to those from *in vivo* derived spermatids. SSCs isolated from prepubertal patients have been propagated *in vitro*, but results are preliminary (Sadri-Arekani *et al.*, 2011). Human prepubertal testicular tissue has also been successfully cultured *in vitro* with survival of the spermatogonial germ cell population, maturation of the somatic cells and formation of the BTB reported (de Michele *et al.*, 2017, 2018). Initiation of spermatogenesis from such tissue, however, has yet to be demonstrated. A recent report has described the generation of sperm-like cells after three-dimensional culture of isolated spermatogonial cells obtained from testis biopsies taken from prepubertal boys undergoing chemotherapy treatment (Abofoul-Azab *et al.*, 2018). This is encouraging for the future, but in this preliminary study, the technique was successful in only one out of six patient biopsies, and the functionality of the sperm-like cells remains to be established.

1.7.2 Protection/Recovery from Chemotherapy Treatment

An alternative option to restoring fertility in the adult would involve protecting the testis during treatment and/or stimulating recovery of the testis through pharmacological protection (reviewed in Allen *et al.*, 2018; Ntemou *et al.*, 2019b). This would provide a non-invasive approach to fertility protection. Several studies have shown the gonadoprotective potential of agents in animal studies. These studies have focussed on adult males, and have mainly investigated the morphology and motility of sperm. For example, Carmely *et al.* (2009) has shown in a mouse model the cytoprotective effect of the immunomodulator AS101 compound against cyclophosphamide-induced testicular damage. Granulocyte colony-stimulating factor (GCSF), which is currently administered to cancer patients to reduce neutropenia, has also shown promise as an agent which can stimulate recovery of the SSC population after chemotherapy treatment as studies in mice have found GCSF to enhance survival of the SSCs following bulsufan treatment (Benavides-Garcia *et al.*, 2015; Kotzur *et al.*, 2017). In addition, the Ntemou *et al.* (2019b) review discusses the potential of miRNA's as a future prospective fertility preservation strategy to

modulate specific pathways to protect and/or restore testis function during/after cancer treatment. To date, however, there are limited studies in prepubertal animals investigating the potential of cytoprotectant agents to protect the testis against chemotherapy-induced toxicity (Table 1.8).

1.7.2.1 Amifostine

Amifostine is an organic thiophosphate that acts as a cytoprotective agent, protecting cells against chemotherapeutic damage whilst having no antitumor activity (Çetingül *et al.*, 2009). This agent is currently used to protect against cisplatin-induced nephrotoxicity in patients with advanced ovarian cancer (reviewed in Hogle, 2007). The active metabolite acts as a ROS scavenger and binds and stabilizes DNA (Spencer and Goa, 1995). This drug has a limited half-life of eight minutes; therefore, *in vivo* studies have focused on pre-treatment of amifostine 15 minutes before chemotherapy treatment. Pre-treatment partially protected the prepubertal rat testis against cisplatin- and doxorubicin-induced testicular damage; this effect may be dependent on the age and schedule of treatment, as a lower dose of amifostine in earlier stages of prepubertal development in rats had no protective effects against doxorubicin-induced damage when tissues were analysed 24 and 48 hrs after treatment (Jahnukainen *et al.*, 2001; Hou *et al.*, 2005). However, prepubertal amifostine pre-treatment before doxorubicin treatment did not maintain fertility in the adult, as DNA damage was found in the sperm of treated animals when analysed 64 days after treatment, which increased the number of arrested embryos in a mating study of adults (100 days old) (Vendramini *et al.*, 2012). An *in vitro* study has also indicated that amifostine has no protective effects against doxorubicin-induced damage in a spermatogonial cell line and an immature Sertoli cell line (Tremblay and Delbes, 2018). Therefore, amifostine may not be a suitable fertility preservation strategy.

1.7.2.2 Carnitine

Carnitine is a quaternary amine found at high concentrations within the epididymis of the male reproductive tract and in spermatozoa. It is acquired through dietary meat and milk, and is also produced by the liver through methylation of lysine and methionine amino acids. This compound has essential roles in determining male fertility, producing energy by transferring long-chain fatty acids into mitochondria, a process required for germ cell maturation, sperm motility and sperm count; it has been shown to have cytoprotective properties whilst having no impact on anti-cancer treatment efficacy (reviewed in Chiu *et al.*, 2004; Sayed-Ahmed, 2010). Partial protection from etoposide-, cisplatin- and doxorubicin-induced morphological damage and apoptotic germ cell death was shown when prepubertal rats were pre-treated with carnitine one-to-two hrs before chemotherapy treatment *in vivo* (Okada *et al.*, 2009; Cabral *et al.*, 2014; Yaman and Topcu-Tarladacalisir, 2018; Aboul-Naga *et al.*, 2020). The mechanism underlying such cytoprotective action is unknown, but may be the result of enhanced DNA repair activity, inhibition of ceramide production and/or reduction in oxidative stress-induced damage as shown in range of different cell types (Andrieu-Abadie *et al.*, 1999; Palmero *et al.*, 2000; Alshabanah *et al.*, 2010). Indeed, improvement in oxidative stress status of the testis following prepubertal pre-treatment with carnitine one-to-two hrs before doxorubicin or cisplatin treatment has been shown in a rodent model (Cabral *et al.*, 2018; Aboul-Naga *et al.*, 2020). An *in vitro* model, however, has found no protective effects against doxorubicin-induced damage when carnitine is administered in spermatogonial or immature Sertoli cell lines (Tremblay and Delbes, 2018). Sertoli cell function may be improved upon carnitine administration, as these cells express carnitine/organic cation transport 2 receptors, with carnitine important in the maintenance of the BTB (Palmero *et al.*, 2000). The recent study by Aboul-Naga *et al.* (2020) investigated further the formation and maintenance of the BTB following prepubertal and pubertal exposure to carnitine two hrs before and on a weekly basis following cisplatin treatment. Carnitine increased the expression of vimentin and occludins, which were reduced with cisplatin treatment alone. Vimentin is a structural protein

that forms the microfilaments, microtubules and intermediate filaments of the Sertoli cells. Occludins protein is expressed within tight junctions that are crucial to BTB integrity. This study clearly showed how carnitine could protect against cisplatin-induced damage not only targeting the BTB, but by also targeting oxidative stress and inflammation.

1.7.2.3 Herbal Remedies

The herbal root, Ginseng has been used in East Asian countries as a traditional Chinese medicine and the intestinal metabolite, known as ginseng intestinal metabolite I (GIM-I), is thought to have multiple pharmacological effects through its antioxidant activity (Zhang *et al.*, 1996). The protective effect against doxorubicin-induced damage has been analysed by Kang *et al.* (2002) in prepubertal mice *in vivo*, where GIM-I was found to partially protect against doxorubicin-induced germ cell damage resulting in testicular morphology comparable to controls that may be the result of enhanced antioxidant activity. GIM-I increases the levels of testis-specific antioxidants which were reduced following doxorubicin treatment. The antioxidant capacity of the leaf extract of the *Moringa oleifera* plant (Moringaceae family) (MOE) has been proposed as a mechanism which confers medicinal properties with this extract (Bharali *et al.*, 2003). MOE (commonly known as drumstick tree or horseradish tree) is native to India and is an ingredient in many traditional Indian dishes. Pre-treatment with ethanol extracts of MOE were found to reduce cyclophosphamide-induced damage to sperm density, integrity and motility with a concomitant increase in antioxidant capacity of the tissue (Nayak *et al.*, 2016). These compound have great potential as a cytoprotective agents with both remedies having anti-metastatic activity and could therefore be added to chemotherapeutic regimens to provide benefit as both a cytotoxic and cytoprotective agent (Hasegawa *et al.*, 1997; Sreelatha *et al.*, 2011). The use of herbal remedies in cancer treatment is however disputed in some scientific communities, as the mechanism of action of these compounds and the potential side effects of usage are not fully understood (discussed in Chapter 7.2).

1.7.2.4 Vitamin C and Curcumin

A study by Tremblay and Delbes, (2018) has investigated the potential of compounds as cytoprotectants based upon their antioxidant activity, including vitamin C and curcumin. Vitamin C can also function by inhibiting apoptosis, while curcumin has additional anti-inflammatory properties. However, in both cases treatment did not reduce the cytotoxic activity of doxorubicin in either spermatogonial or immature Sertoli cell lines. This study therefore indicates that these compounds may not be suitable cytoprotectants against doxorubicin-induced prepubertal testicular damage.

1.7.2.5 Mesenchymal Stem Cells

The use of stem cell therapies in tissue repair to attenuate chemotherapy-induced damage is a growing area of research (reviewed in Rühle *et al.*, 2018). Mesenchymal stem cells are multipotent cells that potentially have the capacity to differentiate into all three cell lineages (ectoderm, mesoderm and endoderm) depending on tissue environment and/or growth factors or media in culture. These cells can be derived from multiple tissues, including bone marrow, skin, vasculature and adipose tissue (reviewed in Ullah *et al.*, 2015).

Mesenchymal stem cells are capable of migration towards damaged tissues, with integration into the testis noted following chemotherapy-induced damage. These mesenchymal stem cells have been shown to differentiate into germ cells at the basement membrane in small numbers of seminiferous tubules (Nayernia *et al.*, 2006). The capacity for these mesenchymal stem cell derived germ cells to complete spermatogenesis is controversial, with some studies finding restoration of full spermatogenesis and fertility (Cakici *et al.*, 2013; Monsefi *et al.*, 2013), whereas others have not (Nayernia *et al.*, 2006; Ghasemzadeh-Hasankolaei *et al.*, 2016). To date, one study has investigated the potential of bone marrow derived mesenchymal stem cells (BM-MSCs) in restoring testis morphology and fertility following doxorubicin treatment in prepubertal rats (Abdelaziz *et al.*, 2019). Injection of BM-MSCs seven days after doxorubicin treatment was able to ameliorate doxorubicin-

induced oxidative stress, atrophy of seminiferous tubules, sperm abnormalities, post implantation loss and fetal growth retardation. The impact of mesenchymal stem cell transplantation on tissue repair may also occur through secretion of paracrine factors such as cytokines, growth factors, signalling lipids, mRNAS and miRNAs. Whether the protective effects of MSCs in the above Abdelaziz *et al.* (2019) study were a result of paracrine factors produced by the MSCs or due to integration of MSCs into the tissue is unknown. Recent research has shown the potential of human umbilical cord mesenchymal stem cell-derived conditioned medium in protecting against cisplatin-induced ovarian damage and infertility with an *in vivo* and *in vitro* model (Hong *et al.*, 2020). This study was also able to identify a number of potential factors involved in this protection, including GCSF. All such studies have so far taken place in animal models, therefore further research and optimization is required before any clinical applications can be discussed. For example, whether these injected mesenchymal stem cells/conditioned medium have the potential to form tumours and/or protect tumours from chemotherapy treatment needs further investigation (reviewed in Rühle *et al.*, 2018).

Table 1.8: Potential cytoprotective agents to protect the prepubertal testis against chemotherapy-induced damage. Updated since publication in Allen *et al.*, (2018)

Compound	Dose	Chemotherapy Drug/Doses	Animal Model/Age	*In vivo* or *In vitro*	Effect	Papers
Amifostine	0.4g/kg	Cisplatin 0.005g/kg	Prepubertal rat (pnd30)	*In vivo*	Partial protection, ↓seminiferous tubule area and ↑ apoptotic spermatogonia and primary spermatocytes comparable to control or amifostine treated.	Lirdi *et al.*, 2008
Amifostine	0.4g/kg	Doxorubicin 0.005g/kg	Prepubertal rat (pnd30)	*In vivo*	Partially protects. Did not protect against DNA damage and negatively impacted on embryo development and pregnancy outcome.	Vendramini *et al.*, 2010, 2012
Amifostine	0.2g/kg	Doxorubicin 0.003g/kg	Prepubertal rat (pnd6)	*In vivo*	No protective effects.	Jahnukainen *et al.*, 2001; Hou *et al.*, 2005
Amifostine	1µM	Doxorubicin 0.01-1µM (24hrs)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 hrs had no impact on cytotoxicity in the Ser-W3 cell line. Co-treatment had no impact on GC-6spg cell line cytotoxicity.	Tremblay and Delbes, 2018
Carnitine	0.25g/kg	Etoposide 0.04g/kg	Prepubertal rat (pnd25)	*In vivo*	Analysed pnd30, 64 & 100. Partial protection, reduction in TUNEL+ cells.	Okada *et al.*, 2009

Carnitine	0.25g/kg/day	Doxorubicin 0.005g/kg	Prepubertal rat (pnd30)	*In vivo*	Analysed pnd64 & 100. Partial protection, ↓TUNEL+ cells and sperm DNA damage. ↑acrosome integrity pre-treatment, no impact on sperm motility and mitochondrial activity. ↓lipid peroxidation and nitric oxide. ↑fertility index and implantation rate improved.	Cabral *et al.*, 2014, 2018
Carnitine	0.25g/kg (1hr & week after)	Cisplatin 5mg/kg	Prepubertal rat (pnd30)	*In vivo*	Partial protection. 24hrs later, ↓testicular damage, ↓TUNEL+ cells and ↑PCNA (proliferating cells) compared to cisplatin. Analysis at pnd90 showed ↑epididymal sperm count and viability. *No carnitine alone treated animals included.	Yaman and Topcu-Tarladacalisir, 2018
Carnitine	0.25g/kg/wks (8 wks)	Cisplatin 5mg/kg	Prepubertal rats (pnd 30)	*In vivo*	Analysed at pnd 37, 60 and 90. Partial protection; testis morphology, oxidative stress (↓MDA), ↑sperm count, motility, viability & ↓sperm DNA damage Partial restoration; hormone levels (T, LH, FSH) and antioxidant capacity (↑CAT) Carnitine; ↑anti-apoptosis (BCL2), chromatin remodelling genes and ↓pro-inflammatory genes (IL-6) treatment. Promoted protamination and blood-testis barrier integrity	Aboul-Naga *et al.*, 2020

Carnitine	10mM	Doxorubicin 0.01-1µM (24hrs)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 hrs or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line. Co-treatment had no impact on cytotoxicity of GC-6spg cell line.	Tremblay and Delbes, 2018
Ginseng Intestinal Metabolite I (GIM-I)	0.05g/kg/day	Doxorubicin 0.05g/kg per day	Prepubertal rat (pnd28)	*In vivo*	Partially protects.	Kang *et al.*, 2002
Moringa oleifera leaves extract (MOE)	25mg/kg	Cyclophosphamide 50mg/kg (single dose)	Prepubertal mice (pnd14)	*In vivo*	Analysed at pnd42. Improved sperm conc to control levels with pre and post MOE treatment. ↓ abnormal sperm head morphology. No change in testis morphology.	Nayak *et al.*, 2016
Vitamin C	40µg/ml	Doxorubicin 0.01-1µM (24hrs)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 hrs or co-treatment no impact on cytotoxicity in the Ser-W3 cell line. Pre-treatment ↓ cell viability GC-6spg cell line (25 or 40µg/ml) Co-treatment at 10µg/ml no impact on GC-6spg cell line.	Tremblay and Delbes, 2018
Curcumin	5µM	Doxorubicin 0.01-1µM (24hrs)	GC-6 spermatogonial and Ser-W3	*In vitro*	Pre-treatment for 24 hrs or co-treatment no impact on cytotoxicity in the Ser-W3 or GC-6spg cell line	Tremblay and Delbes, 2018

			immature SC cell line (rat)			
Bone marrow Mesenchymal stem cells (BM-MSCs)	$2x10^6$ cells on day 7	Doxorubicin 5mg/kg (single dose)	Prepubertal rats (pnd 30)	*In vivo*	BM-MSCs restored testis morphology; ↓testicular atrophy & damage to spermatogenesis, ↑diameter & germinal epithelium thickness Improved sperm parameters; ↑ sperm count and ↓ abnormal sperm ↓ elevated MDA and ↑ antioxidant capacity	Abdelaziz *et al.*, 2019

BM-MSCs; bone marrow mesenchymal stem cells, CAT; catalase, FHS; follicle stimulating hormone, GC; germ cells, LH; luteinizing hormone, MDA; malondialdehyde, SC; Sertoli cells, SSCs; spermatogonial stem cells, T; testosterone.

1.7.2.5 Summary of Research into Fertility Protective Strategies

Fertility preservation for prepubertal boys undergoing gonadotoxic chemotherapy treatment is an on-going field of research with success noted in animal model studies (reviewed in Picton *et al.*, 2015; Kilcoyne and Mitchell, 2019a; Goossens *et al.*, 2020). Testicular biopsies and cryopreservation of prepubertal tissue is currently being undertaken at a number of research centres, although optimization of patient selection and methodology are still required. Transplantation of prepubertal tissue/SSC and *in vitro* propagation of viable sperm are currently being considered for fertility preservation strategies; however, there is limited success with human tissue. Cytoprotective agents could play a major role in the future of fertility preservation strategies, however, research in this area to date is very limited, and has only shown partial protection against chemotherapy-induced damage with treatment with amifostine, carnitine, GIM-I and MOE in animal model studies. The use of BM-MSCs and mesenchymal derived condition medium to restore normal testis morphology is an interesting avenue of research. Overall, despite the promising results outlined, the level of evidence established so far in animal studies in not sufficient for transfer to clinical practise and needs further investigation. In this thesis, additional antioxidants including melatonin, n-acetylcysteine (NAC) and lycopene will be investigated for potential protective effects against cisplatin-, doxorubicin- or vincristine-induced damage with use of GC-6spg cell line and prepubertal mouse testis culture (Chapter 6).

1.8 Conclusions from the Current Literature and Research Prospects

Cancer therapy with a range of chemotherapy agents from different drug classes during childhood have been found to negatively impact upon the prepubertal testis. The resulting damage depends on the compounds used, cumulative dose, administration regimen and age/pubertal status during treatment. Such conclusions have been drawn from both clinical investigations and animal models, including *in vivo* as well as *in vitro* studies, with testicular fragments or primary cell cultures/cell lines representative of the cell types in the prepubertal testis. However, the evidence

for chemotherapy-induced damage to the prepubertal testis is at present incomplete and requires further investigation. Such investigations were undertaken during this PhD project to further elucidate the gonadotoxicity and mechanisms of action of chemotherapeutic drugs that are commonly used in paediatric oncology. With this knowledge, cytoprotective agents that protect the prepubertal testis against chemotherapy-induced damage can be selected/developed and investigated. Multi-drug treatment regimens are almost always employed to effectively treat the cancer and reduce the chance of the cancer developing resistance to the drugs, using chemotherapy drugs from various classes, which therefore have different mechanisms of action. A cytoprotective strategy to protect the prepubertal testis tissue would therefore potentially have to protect against multiple different gonadotoxic chemotherapy drugs, each with different mechanisms of action. Targeting a common mechanism shared by gonadotoxic agents would be an ideal approach to developing a cytoprotective strategy. Certain chemotherapy drugs that are also considered gonadotoxic, including cisplatin and doxorubicin, are known to induce oxidative stress, with the role investigated in relation to other known associated toxicities of these drugs (reviewed in Conklin, 2004; Chen *et al.*, 2007). Oxidative stress results from an imbalance in ROS production and antioxidant capacity, leading to cellular dysfunction/damage and potentially cell death(reviewed in Birben *et al.*, 2012; Sies, 2015). Combatting reactive species/oxidative stress to protect healthy tissues, without interfering with the anticancer action of agents, may be a strategy that could protect across various drug classes. Research into the role of oxidative stress in gonadotoxicity following cisplatin and doxorubicin-induced damage has been conducted in animal model studies in adults, as well as in the prepubertal testis, by examining the protective effects of antioxidants (see Table 1.9 Chapter 1 for prepubertal testis).

1.9 Overall Aims of PhD Project

Improvements in childhood cancer treatment, particularly the use of chemotherapy drugs, has led to increased survival rates, currently around 80% (Miller *et al.*, 2016).

Chemotherapy treatments, however, can damage healthy tissues such as the reproductive tissues, which can lead to infertility in adulthood (Chow *et al.*, 2016). At present, there are no fertility preservation options for young, prepubertal boys undergoing cancer treatment since they are not able to produce mature spermatozoa for sperm cryopreservation (reviewed in Picton *et al.*, 2015; Mitchell *et al.*, 2017; Goossens *et al.*, 2020). Research into alternative fertility preservation strategies are experimental and have been successful in animal models only. One option under investigation includes the use of cytoprotective agents during cancer treatment to protect the prepubertal testis against chemotherapy-induced damage (reviewed in Allen *et al.*, 2018; Ntemou *et al.*, 2019b). To develop such agents, however, a thorough understanding of the gonadotoxicity and mechanism of action of chemotherapy agents are required in relation to the prepubertal testis. This thesis therefore aimed to examine the gonadotoxicity of certain commonly used chemotherapy agents, and to determine whether oxidative stress was involved in the underlying mechanism of action. In addition, antioxidants were tested to determine whether they could protect against chemotherapy-induced testicular toxicity. Two *in vitro* models were used; a mouse prepubertal testis culture model and a rat GC-6spg cell line model that is representative of the SSCs/spermatogonia type A that are found in the prepubertal testis.

The hypotheses and aims of this PhD are as follows,

Hypothesis 1; Newer generations of chemotherapy agents are less gonadotoxic compared to older generations. Newer generations are currently used to replace older generations of drugs in chemotherapy treatment regimens in order to reduce associated side effects that can be dose limiting. Whether these newer generations also diminish associated gonadotoxicity is unknown.

Aim 1; To compare the gonadotoxicity of the second-generation drug carboplatin to the parent compound cisplatin utilizing cultured prepubertal mouse gonads (Chapter 3)

Hypothesis 2; The underlying mechanism of action of cisplatin- or doxorubicin-induced gonadotoxicity to the prepubertal testis involves the production of ROS and oxidative stress, whereas vincristine treatment is not considered to induce oxidative stress nor induce significant levels of damage to the prepubertal testis. Currently, there is a proposed role for oxidative stress in cisplatin-induced nephrotoxicity and doxorubicin-induced cardiotoxicity (reviewed in Conklin, 2004); however, a role in gonadotoxicity in the prepubertal testis is unclear.

Aim 2a; To determine the gonadotoxic impact of vincristine treatment upon the prepubertal mouse testis (Chapter 4)

Aim 2b; To assess the role of ROS and oxidative stress in the underlying mechanism of action of cisplatin-, doxorubicin- or vincristine-induced damage to the prepubertal mouse testes and rat GC-6spg cell line (Chapter 5)

Hypothesis 3; Antioxidant co-treatment protects against cisplatin- or doxorubicin-induced damage to the rat GC-6spg cell line, targeting ROS and oxidative stress. Such antioxidants could therefore be utilized as cytoprotective agents to protect the prepubertal testis against chemotherapy-induced damage

Aim 3; To assess the protective capacity of the antioxidants melatonin, NAC and lycopene against chemotherapy-induced damage in a GC-6spg cell line (Chapter 6)

Chapter 2. Materials and Methods

This chapter contains material and methods where they are relevant to more than one chapter within the thesis. Details specific to methods used in individual chapters are given in the relevant chapter.

2.1 Prepubertal Testis Tissue Culture

2.1.1 Preparation of Media and Culture Plates

All media was prepared in a laminar flow hood under sterile conditions. The dissecting media was composed of leibovitz (L-15) media (Invitrogen, catalogue number: 11415049) supplemented with 3mg/ml of bovine serum albumin (BSA, Sigma, A3311). This was sterilised using Becton-Dickson syringes and syringe filters (Fisher Scientific, 10509821), collected in a centrifuge tube and warmed at 37^{o}C in an oven for at least 1-2 hrs before use.

The culture media was α-MEM (Invitrogen, 22571020) supplemented with 10% knockout-serum replacement (KSR; Fischer Scientific, 10828010). Media was sterilised as described above. One ml of culture medium was required per well to prepare the culture plate, whereas 500µl of medium per well was required to prepare drug media. Media was prepared in advance and placed in the incubator to equilibrate at 34^{o}C, 5% CO_2 for at least 1-2 hrs. Media was either collected in centrifuge tubes (placed in incubator with the lid left ajar to allow for equilibration of media) or aliquoted directly into the plate (see below).

For culture, 24-well plates (Greiner, 662960) were used where 1ml culture medium were added to each well. Sterilised tweezers were used to place a nucleopore polycarbonate membrane (Whatman, 110414) on top of the media to float with the shiny side facing upwards.

2.1.2 Tissue Collection and Culture

To set up a 24-well culture plate, a minimum of two CD1 pnd five mouse pups from a single litter (sample size 'n'=1) were culled through cervical dislocation, with that procedure performed by an animal house technician. Culled pups were dissected in sterile conditions under a flow hood, where the animal was pinned to a corkboard and a ventral incision made with the use of forceps and scissors at the level of the navel to expose the abdomen. The testes, located on either side of the bladder, were removed by cutting the vas deferens and placed within embryo dishes containing pre-warmed dissecting medium (see above 2.1.1). Under a microscope in a laminar flow hood, extra surrounding tissue and the tunica of the testis were dissected away with the use of insulin syringe needles. Pre-culture controls of either whole or half a testis from each litter were transferred into PBS 1X (Fischer Scientific, 10209252) for later fixation (see below 2.3.1). Due to the size of the testis, the tissue cannot be cultured whole since the centre would become necrotic. Therefore the testes were further dissected into ~1mm^3 fragments using a scalpel blade (Altomed, A10136) and randomly transferred to floating membrane using coated drawn Pasteur pipettes. Testis tissue was cultured for four days in total with the addition of chemotherapy agents on the second day of culture for 24 hrs. Chemotherapy drugs were added to culture by replacing 500µl of the 1ml of media within the well with 500µl of drug media at 2X the concentration required; upon addition to the well, this would dilute the drug concentration 1:2 to achieve the desired concentration. Similarly, media in control wells was replaced in the same manner, with fresh drug-free media containing equivalent concentrations of drug solvent. At the end of the 24 hrs drug exposure period, membranes were transferred by use of sterile forceps into freshly prepared plates containing drug-free media (see above 2.1.1). To track proliferation, 15µg/ml BrdU was added for the final 24 hrs of culture. At the end of culture, media was replaced with PBS 1X and transferred for fixation (see below 2.3.1). Overview of culture shown in Figure 2.1. The 'n' number/biological replicate for these experiments refers to litters. Technical replicates per litter, per condition were also included in experiments to act as a backup tissue.

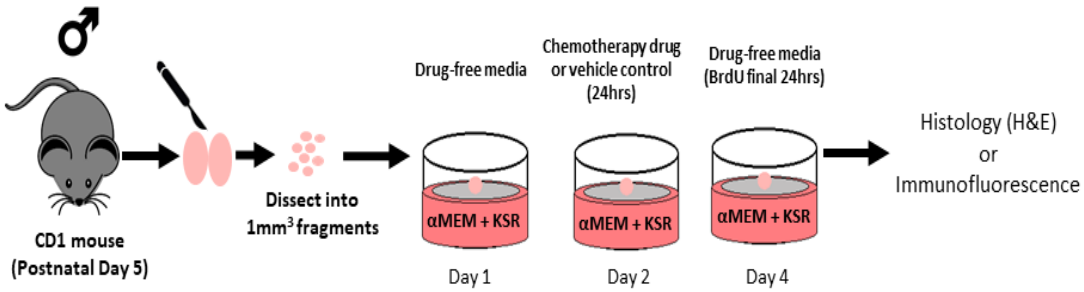


Figure 2.1. Four-day culture system for mouse prepubertal testicular tissue. Testis tissues from postnatal day five CD1 mice were collected, dissected into 1mm^3 fragments and randomly placed on floating membranes in wells containing 1ml αMEM media supplemented with 10% knockout serum replacement. Chemotherapy drugs were added on the 2nd day of culture for 24hrs only. During the final 24hrs of culture, BrdU was added to enable assessment of proliferation. At the end of culture period, tissues were fixed for either histological or immunofluorescence analysis. Image created by C.Allen.

2.2 Chemotherapy Drugs

2.2.1 Powder Forms

At the start of this PhD investigation, powder forms of chemotherapy agents were used and made up in deionized water (Invitrogen, 15230089) or DPBS (Invitrogen, 14190094) as follows; 1mg/ml cisplatin (Millipore, 232120), 10mg/ml carboplatin (Millipore, 216100), 1mg/ml doxorubicin hydrochloride (Abcam, ab120629) and 1mg/ml vincristine sulphate (Abcam, ab120226). Chemotherapy drugs were prepared in sterile conditions under a class II laminar flow hood where 1.5ml Eppendorf tubes were weighed before and after addition of powder and the difference used to calculate how much of the solvent was required to obtain the desired stock concentrations as noted above. Powders were dissolved by vigorous pipetting and vortexing. Eppendorf tubes of stock solutions were sealed with parafilm and kept in the fridge at 4^{o}C for a maximum of one week.

2.2.2 Clinical Preparations

To reduce the risk of exposure of the operator to the chemotherapy drug during preparation of powder drugs, a change was instituted to instead use clinical preparations of drugs, as follows: 2mg/ml doxorubicin (Clinigen, 550921293),

1mg/ml vincristine sulphate (Clinigen, 55790169) and 1mg/ml cisplatin (Clinigen, 50923760). Excipients of drugs were detailed in the data sheets, and included saline solution (doxorubicin and cisplatin), mannitol (vincristine sulphate), water for injection and hydrochloric acid/sodium hydroxide to control pH. Concentrations of saline and mannitol were not provided.

2.3 Histology

2.3.1 Fixation and Agar Embedding

Following culture, the 1X PBS was removed from the culture plates and replaced with either Bouins fixative or 10% neutral buffered formalin (Sigma, HT5014-1CS) for a minimum of one hr and up to 24 hrs. After fixation, the fixative was removed, tissues washed twice with 70% ethanol and placed in 70% ethanol for storage. Due to the small size of the testis tissue fragments, each fragment was placed in the centre of a 3% agarose droplet (Sigma, A1296) (agar prepared with ddH_2O) for support and ease for handling and visualisation when sectioning.

2.3.2 Wax Embedding

Agar blocks containing the testis fragments were placed in cassettes for processing. The cassettes were passed through alcohol solutions of increasing concentrations (70%, 90%, 2x 100%) and immersed in paraffin wax and xylene solution to enable penetration of the paraffin wax into the tissue. Agar blocks were then placed in truncated plastic moulds (Park Scientific), filled with wax and allowed to cool before sectioning. Either Viv Allison or Louise Dunn performed the wax embedding.

2.3.3 Sectioning and Mounting

Wax blocks were removed from their moulds, trimmed down with a razor blade so the bases were flat, and then fused to a heated chuck and placed in water to cool. The surface of the block was further trimmed to centre the testis fragment. The chuck was fixed onto the microtome and orientated so that the block was at right angle to the blade. The whole tissue was sectioned at 5µm thickness and collected in ribbons.

The ribbon was then divided after every third section, floated in a warm water-bath (42^{o}C) and collected using Superfrost Plus slides (Fisher, 10149870) for immunofluorescent staining or twin-frosted slides (Fisher, 12362098) for Haematoxylin and Eosin (H&E) staining. Slides were placed in a drying rack within a 37^{o}C oven overnight to dry.

2.3.4 H&E Staining

Selected slides were put in a staining rack and immersed in xylene for dewaxing of sections for ten minutes (x2), after which the rack was immersed in decreasing concentrations of ethanol for one minute each (100%, 95%, 90%, 70%) to rehydrate the tissues. The slides were also submerged in 70% ethanol lithium carbonate for one minute to remove Bouin's fixative. Slides were then washed for five minutes in tap water, submerged in filtered haematoxylin for three minutes to stain the nuclei and washed in tap water. The slides were dipped in acid alcohol to remove excess haematoxylin, washed in tap water and intensity of the stain checked under a microscope. The slides were then submerged in Scott's tap water solution for three minutes to 'blue up' haematoxylin and washed in tap water for three minutes. Finally, the slides were submerged in filtered eosin for two minutes, briefly washed and submerged in potassium aluminium for two minutes, fixing the stain. After checking intensity of stain, the slides were immersed in increasing concentrations of ethanol (70%, 90%, 95%, 100% x2) to dehydrate the tissues. The tissues were cleared in xylene, mounted with xylene-based mountant DPX (Phthalane Free) (Cellpath, SEA-1300-00A) and covered with a coverslip avoiding formation of air bubbles. Slides were left in a fumehood overnight to dry and imaged with a bright-field microscope (DMLB microscope, DFC480 camera and IM50 software, Lecia).

2.4 Immunofluorescence

2.4.1 Selection of Slides

A maximum of 25 slides were included in a run with slides from each experimental condition included from a single experimental unit/sample/'n'. Depending on the

number of conditions, three sections from the start, middle and end of each tissue were included (variations are stated in the relevant chapters) and placed in a metal slide holder. For every run, a slide was included for a 'no antibody' control where the primary antibody was excluded. Due to the size of the tissue, a diamond pen was used to etch a circle around the sections under a microscope to ease visibility.

2.4.2 Dewaxing, Rehydration and Antigen Retrieval

Slides were dewaxed in xylene for ten minutes (x2) and tissues rehydrated by passing slides through decreasing concentrations of ethanol (100% x2, 95%, 90%, 70%) and washed in PBS 1X. Slides were then transferred into a plastic slide holder and incubated for ten minutes in citrate buffer (sodium citrate, 10mM, pH 6, Fischer) to equilibrate. Antigen retrieval was undertaken through heat-induction by microwaving slides for five-minute bursts (four times each) with the addition of 50ml of ddH_2O in between each burst to prevent drying out of slides due to evaporation. Antigen retrieval ensures that the proteins that are masked by cross-linking during fixation and processing are broken so that the antigen of interest is free to bind to the primary antibody. Slides were cooled for at least 20 minutes and washed in PBS 1X for five minutes before blocking.

2.4.3 Blocking

To contain the blocking/antibody solution on the slide, the area around the section was dried and a small wax ring drawn with a hydrophobic barrier PAP wax pen (Vector labs, H-4000) to create an area that could contain the 50µl of solution. Slides were then dipped in PBST (PBS 1X, 0.1% Triton X-100, Sigma) to permeabilise the cells. To prevent non-specific binding, the tissues were blocked with PBST, 5% BSA and 20% goat serum (Sigma, G6767) as all our secondary antibodies were raised in goat. The slides were incubated with blocking solution for at least one hr in a humidified chamber at room temperature. The chamber was created within a plastic box which contained moist paper towels to create the humidified environment. After blocking, slides were washed in PBS 1X before the addition of the primary antibody.

2.4.4 Primary and Secondary Antibodies

The primary and secondary antibodies were diluted in solution containing PBST, 5% BSA and 10% goat serum with 50µl added to each slide. Commonly used antibodies and dilutions used across multiple chapters in this thesis are shown in Table 2.1. The slides were incubated with the primary antibody solution overnight in the humidified chamber at 4°C. On the following day, the slides were washed in PBST for five minutes x2 on a shaker. The secondary antibody solution was then added to the slides for one hr at room temperature in the humidified chamber with care taken to protect the slides from light to avoid over exposure. Slides were then washed in PBST for five minutes x2 before counter staining.

Table 2.1 Primary and secondary antibodies for immunofluorescence

Primary Antibody	Primary Ab Dilution	Secondary Antibody (diluted 1:500)
Mouse anti-MVH (Abcam, ab27591)	1:200	Alexafluor goat anti-mouse IgG 488 nm or Alexafluor goat anti-mouse IgG 568nm
Rabbit anti-SOX9 (Abcam, ab5535)	1:500	Alexaflour goat anti-rabbit IgG 488nm
Rat anti-BrdU (Abcam, ab6326)	1:500	Alexafluor goat anti-rat IgG 568nm
Rat anti-Ki67 (Invitogen, 14-5698-82)	1:1000	Alexaflour goat anti-rat IgG 568nm

2.4.5 Counterstaining, Mounting and Visualisation

Tissues were counterstained with DAPI (1:5000, diluted in PBS 1X) by flooding the slide with the counterstain and then rinsing in PBS 1X for five minutes. The tissues were mounted with Vectashield hard set antifade medium (Vector Labs, H-1400) with a coverslip on top. Coverslips were sealed by applying nail polish around the edges; slides were placed in a slide holder, covered in tin foil to protect from light and stored

in the fridge at 4^{o}C. Slides were imaged using a fluorescent microscope (Lecia DM4400B on a DCFC360FX camera) with fluorophore settings maintained for imaging all slides from each immunofluorescent run. Images were taken at 20X magnification for analysis; multiple images were often required to capture the whole section.

2.4.6 Analysis

Image analysis was undertaken using Image-J/Fiji software (JAVA), with assessor blind to experimental conditions: this was achieved by having images randomised using RandomNames generator software (HowToGeek.com, written by Jason Faulkner). Cell densities were calculated either through manual counting of positive cells per area of section/seminiferous tubule or by semi-automative analysis where area of fluorophore expression was analysed per section/seminiferous tubule area (expressed as %). The scale on ImageJ was set for the 20X images where 104 pixels represented 50µm. The areas were calculated through use of freehold tool on ImageJ, which was used to draw around the edge of the section or to draw around the edge of the seminiferous tubules using the PMC layer for reference. For semi-automative analysis, the colour channels were split for each image to separate out the fluorophore channels, where fluorophore expression was analysed as an area. For each run of an immunohistochemistry analysis, a threshold for intensity of expression was set to pick up the fluorophore expression; this threshold level was then used for all analysis within that run. Where multiple images were taken to capture the whole section (see 2.4.6), results from each section part were added together to give cell densities per total section. Where multiple sections of a tissue were analysed (see 2.4.1 above), an average was used to give a result per tissue/condition. Representative images used in the thesis were chosen from across culture runs: in those instances, fluorophore intensity was sometimes altered in order to highlight expression for illustrative purposes.

2.5 Age-Matched Controls

To compare cultured testis fragments to *in vivo* testes, whole testes from age-matched controls were collected (see 2.1.2) and fixed (see 2.3.1) ('n'=6, tissue collected from six separate litters). This includes CD1 pups at pnd five (start of culture) as well as pnd eight and nine (end of four-day culture). These tissues were compared to cultured control testis fragments that were randomly selected from six previous cultures. Germ cells (MVH+) and Sertoli cells (SOX9+) were observed through immunofluorescence (see 2.4), with proliferation (Ki67+) assessed. Density of germ cells per seminiferous tubules were determined through semi-automative analysis (total germ cell density) or manual counting (proliferating germ cell density). This was performed by Emma Guy. Density of Sertoli cells could not be determined due to differences in protein expression levels of SOX9. Data are shown in Appendix B. Data highlight the structural similarities of cultured testis to *in vivo* age-matched controls, indicating that this culture is a good model with which to study testis development. The density of germ cells in culture controls were similar to those of pnd8/9 (end of four-day culture), however the density of proliferating germ cells were reduced *in vitro*, which may be an effect of culture.

2.6 GC-6spg Cell Culture

2.6.1 Overview

Dr. Géradline Delbès (Institut national de la recherche scientifique, Université du Québec, Canada) and Prof. Ans van Pelt (University of Amsterdam, Netherlands) gifted the GC-6spg cell line to Prof Rod Mitchell at passage 53, which they had been found to be the passage at which the cells were most stable. The GC-6spg cell line was created in order to be representative of spermatogonial type A/ spermatogonial stem cells, with originating cells initially isolated from 8-11 weeks of age, Vit A deficient rats (van Pelt *et al.*, 2002). The isolated population of A_s, A_{pr} and A_{al} spermatogonia were transfected with SV40 large T antigen and expressed undifferentiated spermatogonia germ cell specific markers, Hsp90α and Oct-4. All work with this cell line was undertaken in a class II laminar flow hood. Cultures were

tested for presence of mycoplasma using a Lonza MycoAlert Mycoplasma detection kit (cat.no: LT07-318) with 1ml of our cell culture media taken from cultures of at least 80% confluence. This was performed by Theresa O'Connor. These cells were cultured at 32^{o}C, 5% CO_2.

2.6.2 Culture Media

The culture media for the GC-6spg cell line was prepared as described in Table 2.2. The volumes stated are to prepare 150ml of culture medium. The growth factors added were purchased in powder form, stock concentrations prepared, aliquoted and stored as recommended in the data sheets. This includes platelet-derived growth factor-BB human (PDGF) (Sigma, P4306) reconstituted in ddH_2O to produce stock concentrations of 0.1mg/ml, forskolin (Sigma, F6886) reconstituted in DMSO to stock concentration of 5mg/ml, LIF human recombinant animal free (Sigma, GF342) reconstituted in 0.1% BSA solution (BSA dissolved in ddH_2O) to prepare 0.1mg/ml stock, fibroblast growth factor basic protein human recombinant (bFGF) (Millipore, GF003) reconstituted in 0.1M PBS to prepare 0.1mg/ml stock and β-estradiol (Sigma, E2758) reconstituted in 2% ethanol/MEM to prepare 20µg/ml stock. The prepared media was sterilised with use of bottle top vacuum filters (Corning, 431161) and equilibrated to culture conditions in incubator at 32^{o}C, 5% CO_2 before use. The complete culture media stock was stored in the fridge when not in use and kept up to seven days.

Table 2.2: Reagents to make 150ml of GC-6spg cell culture medium.

Reagents (working conc.)	Volume / Mass	Product Information
MEM	136.51ml	ThermoFisher: 11095080
Non-Essential Amino Acids (1X)	1.5ml	ThermoFisher: 11140050
HEPES (15mM)	2.25ml	ThermoFisher: 15630106
Gentamicin (40µg/ml)	0.6ml	ThermoFisher: 15710049
Penicillin/Streptomycin (1X)	1.5ml	ThermoFisher: 15140122
L-glutamine (4mM)	3ml	ThermoFisher: 25030024
FBS (2.5%)	3.75ml	ThermoFisher: 10270106
G418 (200µg/ml)	0.6ml	ThermoFisher: 10131035
Sodium Bicarbonate (0.12%)	0.18g	Sigma: S5761-500g
bFGF (2ng/ml)	2.4µl	Sigma: GF003
PDGF-BB (10ng/ml)	15µl	Sigma: P4306-10µg
Forskolin (20µM)	250µl	Sigma: F6886-10mg
β-estradiol (1nM)	2.1µl	Sigma: E2758-250mg
Human Recombinant LIF (10ng/ml)	15µl	Millipore: GF342

2.6.3 Freezing Down

To cryopreserve the cell line, cells were frozen down in complete culture media supplemented with 5% DMSO (Sigma, D2438) as cryoprotectant. After preparation, the cryopreservation media was placed on ice to equilibrate to a reduced temperature as the cells were frozen through uncontrolled slow-freeze method. Cells were sub-cultured as described in 2.6.5 with the exception that the cells were placed in cryopreservation media. 1ml of the cell suspension containing ~2 million cells were aliquoted into cryopreservation vials (Scientific Laboratory Supplies, 368632K) and placed on ice. Cell line, passage, date and cell number were stated clearly on the vials. Vials were kept overnight at -80^{o}C before transferring to liquid nitrogen for long-term storage.

2.6.4 Resuscitation of cells

Vials of the GC-6spg cell line were stored in liquid nitrogen and resuscitated by fast thaw, where vials were placed in a water bath set at 37^{o}C for five minutes. To the thawed cells, 9ml of medium was added to dilute the cryoprotectant and cells were spun down in centrifuge at 500rpm for ten minutes. The supernatant was then removed and the cell pellet placed in 1ml of fresh culture medium. Cell number and viability were determined through use of Cell Countess II automated cell counter (Life Technologies, 2185A16090274) where 15µl of the sample was diluted in 15µl trypan blue (Sigma, T8154). Two readings were taken from the sample and averaged. Vials with cell viability below 80% were discarded. To maintain our stores of the cell line, cells were initially grown in T75 flasks (Merk, C7231-120EA) with cells plated at 19,000 cm^2 density. Cells at this density would reach confluence after five days culture. The culture medium was replaced every two-three days.

2.6.5 Sub-Culturing/Passage

Once confluence was reached, the culture medium was discarded removing all traces of serum that contains a trypsin inhibitor. To dissociate cells from the culture flask, 2-3ml of trypsin-EDTA 0.05% (Thermofisher, 25300054) was added and cells checked for dispersal under an inverted microscope (Leica, wetzlar GmBH DMIL Type 090-135.001). Flasks were placed in 37^{o}C incubator to aid in dispersal. Once cells had fully dissociated, 7-8ml of culture media was added to the flask to stop the reaction. Cells were spun down and cell number/viability determined as described above. Cells were either plated in new cell culture flask (T75) or frozen down for long-term storage as described above.

2.6.6 Plating and Culture for Experiments

The GC-6spg cell line was used at passage 62 throughout this study with cells that were newly resuscitated as previously described (2.6.4). Cells were seeded at 50,000 cm^2 density in 100µl aliquots into 96-well plates (Greiner, 655090), excluding the wells around the rim of the plate to reduce any potential edge effects with

evaporating media. Blank wells with no cells were also included to obtain background reference values for all experiments. Cells were incubated at 32°C, 5% CO_2 and cultured for two-days until cells were ~80% confluent (assessed under an inverted microscope). Chemotherapy drugs were prepared as previously described (see 2.2) and diluted in fresh culture media to desired concentrations. The cultured media in the plates was discarded and replaced with drug culture media. For control and blank wells, culture media was replaced by fresh media with equivalent concentrations of the drug solvent included. Each culture run/experiment was considered an 'n' of one/biological replicate, within each plate there was a minimum of three and up to ten wells per condition that acted as technical replicates. In experimental conditions, cells were exposed to the chemotherapy drugs for 48hrs.

2.7 MTT Assay

2.7.1 Overview

The MTT assay is a well-established colorimetric assay that is commonly used to determine cell viability through production of formazan product indicative of viable cells (Mosmann, 1983). It has been used here to study cytotoxicity of chemotherapy agents on the GC-6spg cell line at the end of each experiment. This assay utilizes the ability of viable cells to convert the colourless tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a purple formazan product which can be measured through absorbance. This reaction is dependent on active mitochondrial dehydrogenase enzymes which cleaves the tetrazolium ring, producing the coloured product. Preparation and use of this agent was performed under a class II laminar flow hood.

2.7.2 MTT Addition & Solubilisation

MTT was purchased in powder form (Sigma, M5655) and made up in DPBS to produce stock solutions of 5mg/ml which were filtered sterilised (as described in 2.1.1), aliquoted and stored at -20°C. MTT is light sensitive; care was taken to reduce exposure to light throughout the experiment. At the end of the cell culture

experiments, drug media was removed and MTT added at 0.5mg/ml concentration in 100µl of fresh culture media. Plates were incubated with the MTT media for three hrs to allow for uptake of MTT and formation of formazan crystals. Following the incubation period, the MTT was carefully removed from the wells, leaving behind any crystals that had formed. A solubilisation solution containing 1:1 isopropanol and DMSO was prepared and 50µl added to each well. The plates were placed on a shaker for five minutes to aid in the solubilisation of the crystals to produce a purple solution. The absorbance of the solution was read using a microplate reader (Promega, GloMax Explorer system TM433) with 560nm filter.

2.7.3 Analysis

To determine the cell viability for each condition as a measure of cytotoxicity, the absorbance (abs) values for the technical replicates/wells were averaged to give a single value for each condition in that culture run ('n'=1). This single value was plotted as an individual data point. For each condition, the average abs values of the blank wells were subtracted to account for any background abs providing corrected abs values for each condition. If more than two culture plates were required to provide enough wells for the full experiment, controls were included in each plate and the average from the plates taken to provide a control value for the whole experiment. The cell viability was determined by dividing corrected abs values for each condition by the corrected control abs value to give cell viability as a % of abs value per control (Fig. 2.2). The control cells in each experiment were deemed 100% viable: these are represented as a dashed line in graphs.

$$Cell\ Viability\ (\%) = \left(\frac{Condition\ X\ \ Abs\ Value - Blank\ Abs\ Value}{Control\ Abs\ Value - Blank\ Abs\ Value}\right)\ x\ 100$$

Figure 2.2. Equation for the analysis of absorbance data from MTT assay to determine cell viability. X is representative of the different culture conditions under investigation.

2.7.4 Optimization

The MTT assay was optimized for culture with the GC-6spg cell line following exposure to either staurosporine (Abcam, ab120056) or doxorubicin for 24 hrs. Staurosporine is a protein inhibitor that induces apoptosis and was used as a positive control drug within these experiments. Overall, the optimal absorbance values for analysing an MTT assay for cytotoxicity are within 0.2-0.4 abs of control cells: here, this was achieved through culturing the cell line until 80% confluence was reached before the addition of the cytotoxic agents. To obtain the desired abs of control cells, further culture conditions were optimized including the concentration of MTT added to the cells as well as the exposure period to the MTT. High concentrations of MTT can be toxic and reduce cell viability, therefore 0.5mg/ml and 1mg/ml were tested (Fig 2.3A). Since no difference was found between the two, further experiments were undertaken with 0.5mg/ml MTT. The incubation period with the MTT reagent was also tested comparing three hrs or five hrs exposure periods, with no variance found: three hrs incubation with the MTT 0.5mg/ml reagent was therefore used in subsequent experiments (Fig 2.3B). The culture media prepared for experiments contains the phenol red indicator that could contribute to the abs values, since phenol red has the same abs spectra as the formazan product. Comparison of cell viability with cells cultured in media with phenol red MEM or non-phenol red MEM (Thermofisher, 51200038) was therefore undertaken to rule out the phenol red contributions to abs values, however, no difference in abs was noted and therefore original phenol red MEM was used for subsequent studies (Fig 2.3C).

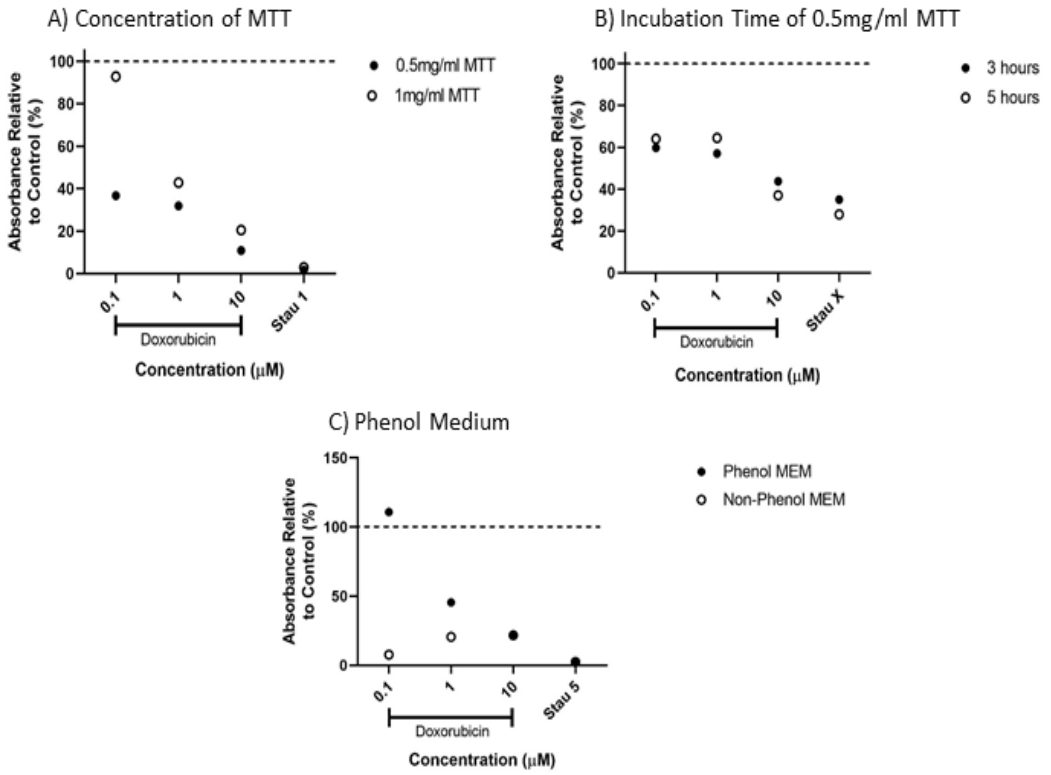


Figure 2.3 Optimization of MTT assay following 24hrs exposure to doxorubicin or staurosporine. A) Concentration of MTT reagent tested comparing 0.5mg/ml (filled dot) or 1mg/ml (open dot). B) Comparison of incubation times of 3hrs (filled dot) or 5hrs (open dot) 0.5mg/ml MTT reagent. C) Comparison of cell viability of cells cultured in phenol red MEM (filled dot) or non-phenol red MEM (open dot). Experiments performed once with cell viability calculated as per Fig 2.2 except for (B) where absorbance values were too low to control for blank abs, this occurred during optimization only. 'n'=1 since one culture run was performed, the data points represent the average from the technical replicates.

Optimization of exposure time to the chemotherapy agents was also undertaken to ensure that the chemotherapy drugs under investigation could be analysed for cytotoxicity at the same time point with concentrations that were within stated patient serum levels. Cell viability was therefore analysed at different time points to determine optimum timeframe for analysis. At 16 hrs post-exposure, there was no clear effect of cisplatin or vincristine exposure, whereas for doxorubicin the cell viability was reduced to around 60% at that timepoint (Fig4.4A). Further analysis of

cisplatin and vincristine at 30, 40, 48 and 72hrs showed effects of drug exposure after 40hrs (Fig2.4 B, C). For our investigation, 48hrs time point was chosen based on these findings. Further optimization of doxorubicin was required to determine whether this drug could be studied at the chosen 48hrs time point with reduced concentrations to enable comparisons with cisplatin or vincristine (Fig 2.5 D,E).

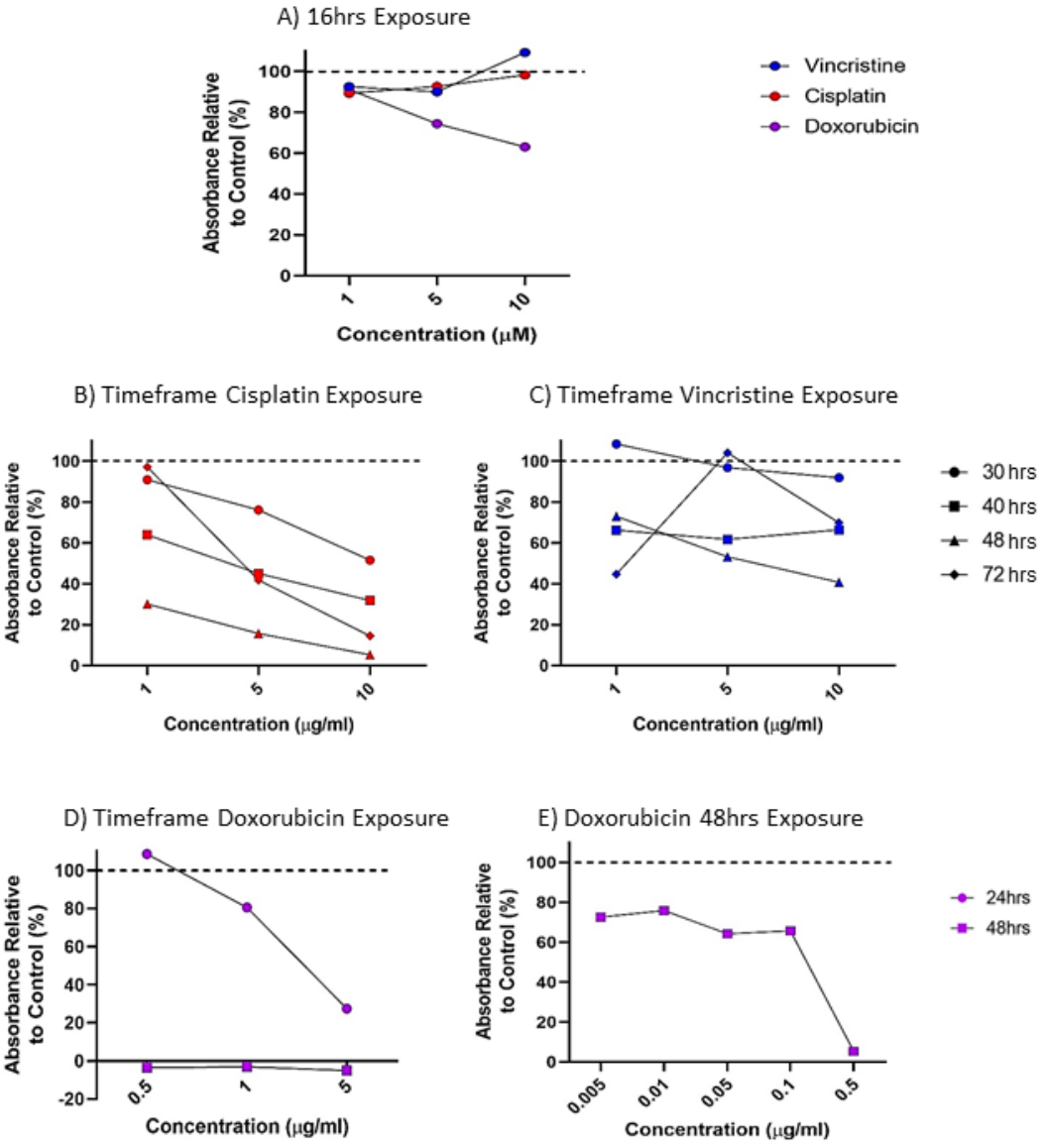


Fig 2.4. Determining the timeframe for exposure to the chemotherapy agent's cisplatin (red), doxorubicin (purple) or vincristine (blue) to observe cytotoxic effects. A) Cell viability of GC-6spg cell line after 16hrs of cisplatin, doxorubicin or vincristine treatment. Cell viability following 30-72hrs of cisplatin (B) or vincristine (C) treatment and 24-48hrs of doxorubicin (D,E) treatment. 'n'=1 since one culture run was performed, the data points represent the average from the technical replicates.

2.8 CellEVENT Probe

Apoptosis was analysed within the cell culture system using the CellEVENT probe (Thermofisher, C10423) that produces a green fluorescent signal upon activation of CC3/7. This probe is cell-permeable and is composed of an amino acid peptide (DEVD) conjugated to a nucleic acid binding dye which is non-fluorescent in this state. Upon activation of caspase 3/7, the DEVD peptide is cleaved from the nucleic acid binding dye, freeing the dye to bind to the DNA and produce a nuclear fluorescent signal that is read/observed within the absorption/emission spectrum of ~502/530nm (ThermoFisher, 2020). This probe was used under a class II laminar flow hood and protected against light at all times.

2.8.1 Addition of Probe

The cells were seeded as previously described (2.5.6) in 96-well black polystyrene plates with a clear flat bottom to minimize background fluorescence. The CellEVENT probe as purchased was diluted in DMSO at 2mM stock concentration, aliquoted and stored at -80^{o}C for long-term storage. The probe was added to the culture at the time of drug addition, where the probe was diluted to a working concentration of 2µM in freshly prepared control/drug culture media. Additional technical controls included blank wells with control and probe-containing medium to take into account any background fluorescent signal from the media. Wells with control cells with no probe but equivalent concentrations of DMSO were also included to check for auto-fluorescence throughout the experiment.

2.8.2 Measurement of Fluorescent Signal

The fluorescent signal was read with a microplate reader (ex. 520nm, emi. 580-640nm) at the desired time points with the plate/lid rim wrapped in parafilm to maintain a sterile environment whilst undertaking the reading. The plates were incubated at 32^{o}C, 5% CO_2 during the intervening time points. At the end of the culture period, the cells were analysed for cell viability using an MTT assay (see 2.7).

2.8.3 Analysis

The fluorescent signals for technical replicates/wells for each condition were averaged to give a single value to which the average fluorescent signal of the blank wells were subtracted to account for fluorescent signal of the medium. The data were shown as relative fluorescent units.

2.8.4 Optimization

The concentration of the fluorescent probe to be used within our culture system was optimized by following the guidelines outlined (ThermoFisher, 2020). Concentrations of 2 and 5µM CellEVENT were assessed after 5 hrs of staurosporine treatment (Fig 2.5 A, B). The fluorescent signal was analysed with a microplate reader (as described above) as well as with an epifluorescent microscope (NIKON ECLIPSE TS100), imaging the cells with brightfield and a green filter. Cells were also checked for auto-fluorescence by including wells with no probe added; showing control and stauropsorine-treated cells themselves do not fluoresce. Based upon these observations, 2µM CellEVENT and analysis with a microplate reader was chosen and used throughout this investigation as the established method.

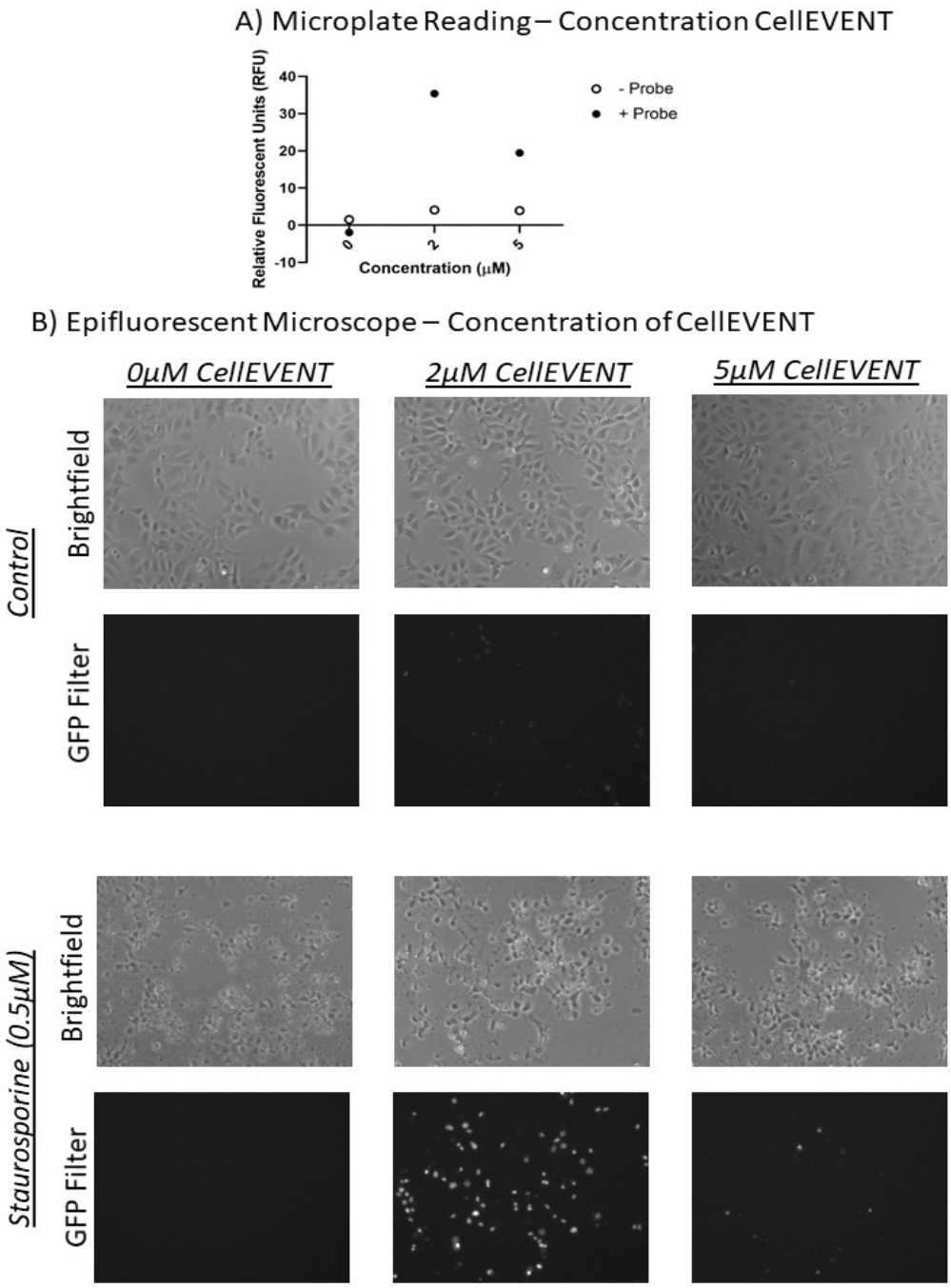


Figure 2.5 Optimization of concentration of CellEVENT probe for detection of cleaved caspase 3/7. CellEVENT concentrations of 2 and 5µM were tested using GC-6spg cell line treated with staurosporine for 5hrs. Cells were also assessed for auto fluorescence of control cells and cells treated with staurosporine. (A) Fluorescent signal detected with microplate reader with cells in the presence (filled dot) or absence (open dot) of CellEVENT probe. (B) Images taken on epifluorescent microscope with Brightfield and green filter. 'n'=1 since one culture run was performed, the data points represent the average from the technical replicates.

2.9 Statistics

Data were analysed and graphs prepared using GraphPad version 8.4.0. Statistical analysis was performed where 'n' was equal to or greater than four. Data was first assessed with descriptive statistics that gave the mean, median, standard deviation and range for the data collected from each experimental condition. The data was also visually assessed by plotting a quantile-quantile (QQ) plot, which plots the quantiles of the data (x-axis) against the predicted quantiles of a normal distribution (y-axis) (see Appendix C). Based upon the descriptive statistics and whether the data plotted in the QQ-plot roughly aligns with the straight line, I assessed whether the data was normally distributed. If I considered the data to not follow a normal distribution, a Shapiro-Wilk test was performed to confirm my observations where $p<0.05$ deemed the data did not follow a normal distribution and would require analysis with a non-parametric test.

For dose-response experiments with cultured tissue and cell culture work with co-treatment of antioxidants, for normally distributed data, a one-way ANOVA was performed, and where a significant difference was found, groups were compared to a 'control' with a post-hoc Dunnett test. For data that was not normally distributed, a non-parametric Kruskal-Wallis test was performed, and where a significant difference was found, a post-hoc Dunns test was used to compare groups to a 'control'. Statistical significance was set at $p<0.05$.

For the dose-response of chemotherapy agents/antioxidants effects on the GC-6spg cell line, normally distributed data were compared with a one-sample t-test to the hypothetical control value of 100%. For non-normally distributed data, a one-sample Wilcoxon test was used to compare data to hypothetical control value of 100%. Where $p<0.05$, the effects at that concentration was deemed significantly different from the control (see at 100%). Data was graphed with the control represented by a dotted line at 100%.

Chapter 3. Comparative Gonadotoxicity of Cisplatin and Carboplatin in Prepubertal Gonads

3.1 Introduction

Current options to preserve fertility for prepubertal boys undergoing chemotherapy treatments are limited and considered experimental, as discussed in Chapter 1 (see 1.7). Examples of such approaches include testicular biopsy and cryopreservation of prepubertal testis tissue, with restoration of fertility theoretically achieved in adulthood through re-transplantation of prepubertal tissue/cells or *in vitro* spermatogenesis to produce mature spermatozoa (reviewed in Kilcoyne and Mitchell, 2019a; Goossens *et al.*, 2020). In addition, cytoprotective agents could be administered during cancer treatment to protect the prepubertal testis from chemotherapy-induced damage: this strategy is investigated further in Chapter 6. Modifying the treatment regimen to include chemotherapy agents that result in less gonadotoxic side effects, whilst maintaining efficacy as an anti-cancer therapy, is also an option to reduce the impact on the prepubertal testis (Viviani *et al.*, 1985; reviewed in Kilcoyne and Mitchell, 2019a). This approach, however, depends upon a thorough understanding of the gonadotoxicity and underlying mechanism of action of chemotherapy agents in relation to the prepubertal testis, which is lacking: that topic will be explored in Chapters 4 and 5. Data from clinical and animal model studies show that chemotherapy agents from the alkylating and alkylating-like drug class are gonadotoxic, depending upon cumulative dose; this includes cisplatin from the platinum alkylating-like drug class (see 1.5.7) (reviewed in Tian En *et al.*, 2020). One other strategy that has been explored is to develop newer generations of these effective 'toxic' agents: these later generations are often reported to have reduced side effects, whilst still preserving their anti-cancer activity (reviewed in Falzone *et al.*, 2018).

In this chapter, the gonadotoxicity of different generations of drugs from the platinum alkylating-like drug class are compared to determine whether newer generations, which have been developed to reduce associated toxicity, are less

gonadotoxic and could therefore be employed as a fertility preservation strategy. Cisplatin (first-generation) and carboplatin (second-generation) are compared using mouse prepubertal culture models for the testis and ovary that have previously been utilized within our laboratory (Morgan *et al.*, 2013; Lopes *et al.*, 2016; Smart *et al.*, 2018). In general, carboplatin is considered to have the same therapeutic index as cisplatin, but with reduced associated toxicities (Ozols *et al.*, 2003; Shah *et al.*, 2018), however this is not the case for all malignancies (Horwich *et al.*, 1997). Compared to cisplatin, carboplatin gonadotoxicity is unknown in relation to the prepubertal reproductive system, with few clinical studies to date investigating the impact of carboplatin treatment during childhood cancer (Reinmuth *et al.*, 2013; reviewed in Tian En *et al.*, 2020). Reinmuth *et al.* (2013) study indicated a potential trend towards infertility following carboplatin treatment, however results did not reach statistical significance, and were based on self-reporting of fertility issues. Based upon the current assumptions, I hypothesised that carboplatin would be less gonadotoxic than cisplatin to mouse prepubertal reproductive tissues and could therefore be used to reduce gonadotoxic damage during cancer treatment.

3.1.1 Publication and Copyright

Findings from this study are published as;

Caroline M Allen, Federica Lopes, Rod T Mitchell, Norah Spears, Comparative gonadotoxicity of the chemotherapy drugs cisplatin and carboplatin on prepubertal mouse gonads, Molecular Human Reproduction, Volume 26, Issue 3, March 2020, Pages 129–140, https://doi.org/10.1093/molehr/gaaa008

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3.1.2 Candidates Contributions

I undertook the work presented in this paper at the start of this PhD programme and was therefore involved/responsible for the design of the study, performing the experiments, analysing the data and writing the paper. For further details, see 'Authors Contributions' at end of paper. Approaches to methodology and statistical analysis were altered during the course of this PhD based upon the lessons learned during the process of performing these experiments and writing the manuscript, this will be discussed (see 3.3.2).

3.2 Paper

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molecular human reproduction

ORIGINAL RESEARCH

Comparative gonadotoxicity of the chemotherapy drugs cisplatin and carboplatin on prepubertal mouse gonads

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ABSTRACT: The treatment of childhood cancer with chemotherapy drugs can result in infertility in adulthood. Newer generations of drugs are developed to replace parent drugs, with the potential benefits of less toxic side effects. For platinum alkylating-like drugs, in contrast to the parent compound cisplatin, the newer-generation drug carboplatin is reported to have reduced toxicity in some respects, despite being administered at 5–15 times higher than the cisplatin dose. Whether carboplatin is also less toxic than cisplatin to the reproductive system is unknown. Here we compare the gonadotoxic impact of cisplatin and carboplatin on female and male mouse prepubertal gonads. *In vitro* cultured CD1 mouse ovaries or testis fragments were exposed to either cisplatin or carboplatin for 24 h on Day 2 of culture and analysed by Day 6. A dose response for each drug was determined for the ovary (0.5, 1 & 5 μg/ml cisplatin and 1, 5 & 10 μg/ml carboplatin) and the testis (0.01, 0.05 & 0.1 μg/ml cisplatin and 0.1, 0.5 & 1 μg/ml carboplatin). For the ovary, unhealthy follicles were evident from 1 μg/ml cisplatin (73% unhealthy, $P = 0.001$) and 5 μg/ml carboplatin (84% unhealthy, $P = 0.001$), with a concomitant reduction in follicle number ($P = 0.001$). For the testis, the proliferating germ cell population was significantly reduced from 0.05 μg/ml cisplatin (73% reduction, $P = 0.001$) and 0.5 μg/ml carboplatin (75% reduction, $P = 0.001$), with no significant impact on the Sertoli cell population. Overall, results from this *in vitro* animal model study indicate that, at patient equivalent concentrations, carboplatin is no less gonadotoxic than cisplatin.

Key words: chemotherapy / fertility preservation / ovary / follicles / testis / spermatogonia / tissue culture / gonadotoxicity

Introduction

Childhood cancer survival rates have markedly increased in recent decades, with the current 5-year survival rate across all childhood cancers above 80% (Miller *et al.*, 2016). This improvement can be attributed in large part to the increasing use of chemotherapy drugs in treatment regimens since their introduction to clinical practice in the early 1940s (Devita and Chu, 2008), along with targeted therapies such as radiotherapy, immunotherapy and surgery. Multiple chemotherapy agents have been developed targeting malignant tumours through various mechanisms, with treatment most often combining different classes of drugs (Lind, 2011). However, damage to healthy tissues can arise as a side effect of chemotherapy treatment, since these cytotoxic drugs can target normal cells as well as malignant ones. It is becoming increasingly apparent that a vast majority of childhood cancer survivors are living with long-term chronic health conditions as a result of the treatment they received (Oeffinger *et al.*, 2006; Hudson *et al.*, 2013; Robison and Hudson, 2014; Bhakta *et al.*, 2017). The impact upon future fertility has been extensively studied, and there are now clear links between the use of alkylating chemotherapy agents in treatment regimens for childhood cancers and subsequent impairment of fertility (Anderson and Wallace, 2016; Stukenborg *et al.*, 2018; Newton *et al.*, 2019). Indeed, a study by Chow *et al.* (2016) has shown a reduced fertility rate for male and, to a lesser extent, female survivors of childhood cancer in comparison to their siblings. In short, it is clear that certain chemotherapy agents damage the reproductive systems of those children undergoing treatment. The most commonly used drugs have been broadly classified according to their predicted risk to future fertility, although these classifications are a matter of debate (Wallace *et al.*, 2005; Wyns *et al.*, 2010; Wallace, 2011). Many studies are based

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on historic treatment protocols that may be outdated and also more aggressive in comparison to current oncology regimens, with newer generations and combinations of drugs considered to be more effective with reduced side effects. As a result, more information is needed on these newer protocols and generations of drugs in order to determine the risk of subsequent infertility for today's patients.

Platinum alkylating-like chemotherapy drugs are commonly used in paediatric oncology treatment regimens for multiple different types of cancer, including germ cell tumours, carcinomas, lymphomas and sarcomas (Dasari and Tchounwou, 2014). Development of these platinum-containing compounds has resulted in different generations including cisplatin (1st generation) and carboplatin (2nd generation) (Weiss and Christian, 1993). These drugs are composed of doubly charged platinum ions surrounded by four ligands: two amine groups and two 'leaving groups' (Desoize and Madoulet, 2002) (Fig. 1). Once inside the cell, the drugs are hydrolysed to form active metabolites that bind to and damage cellular DNA, RNA and proteins, resulting in activation of cell death pathways. Toxicity is mainly attributed to DNA damage where the active metabolites bind to purine bases resulting in cross-linking of the DNA (Dasari and Tchounwou, 2014). However, other molecular mechanisms have been linked to the clinical activity of these drugs, including induction of oxidative stress, modulation of calcium signalling and activation of cellular pathways (Dasari and Tchounwou, 2014). Cisplatin is associated with many toxic side effects including emesis, nephrotoxicity, neurotoxicity, myelosuppression, immunosuppression and ototoxicity; together, these side effects can limit the use of cisplatin in treatment protocols (Florea and Büsselberg, 2011). Cisplatin has been broadly classified as gonadotoxic for paediatric patients (Wyns et al., 2010; Chow et al., 2016), a categorisation that has been backed up by animal model studies (reviewed in Morgan et al., 2012; Allen et al., 2018). Newer generations of this class of drugs have been developed with reduced toxicity; so far, carboplatin is the main drug which has been widely and successfully used as a replacement of cisplatin, although only for certain cancers (Weiss and Christian, 1993; Go and Adjei, 1999). Pharmacologically, carboplatin is 8–45 times less potent than cisplatin for certain cancers and is more stable, possibly due to reduced aquation rates and DNA-damaging effects (Micetich et al., 1985; Knox et al., 1986; reviewed in: Ruggiero et al., 2013; Dasari and Tchounwou, 2014) (Fig. 1). As a consequence, clinically, carboplatin is administered at higher doses in order to be as effective as cisplatin (Hongo et al., 1994; Dasari and Tchounwou, 2014). From published paediatric patient doses, the administered dose of carboplatin can range from 5–15 times higher than the administered dose of cisplatin (Crom et al., 1981; Dominici et al., 1989; Madden et al., 1992; Murry et al., 1993; Newell et al., 1993; Riccardi et al., 1994; Peng et al., 1997; Erdlenbruch et al., 2001; Clerico et al., 2006; Veal et al., 2007). So far, few studies have focused upon the toxicity of newer generations of platinum alkylating-like agents, although carboplatin has been reported to reduce damage to the kidneys in comparison to cisplatin (Canetta et al., 1985). However, myelosuppression and emesis remain associated side effects of both drugs (Canetta et al., 1985). As with cisplatin, carboplatin is currently considered as gonadotoxic based on current classification systems (Wallace et al., 2005; Wyns et al., 2010; Lambertini et al., 2016), although to the best of the authors' knowledge, no studies have focused specifically on the gonadotoxicity of carboplatin in paediatric patients or in prepubertal animal models.

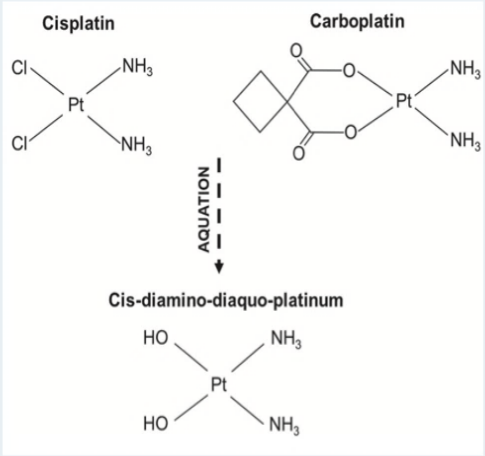


Figure 1 Molecular structure of platinum-alkylating chemotherapy agents and active metabolites formed following aquation of parent drug. Cisplatin and carboplatin share a similar chemical structure with a core platinum ion surrounded by two 'amine groups' and two 'leaving groups'. Cisplatin and carboplatin differ in the leaving groups as shown: cisplatin is composed of chloride ion leaving groups whereas carboplatin contains cyclobutane-decarboxylate leaving groups. Once inside cells, the leaving groups are displaced by water forming aquated active metabolites. The chloride leaving groups of cisplatin readily dissociate whereas the cyclobutane-decarboxylate groups have reduced aquation rates requiring an esterase to dissociate from the parent compound. As a result, cisplatin and carboplatin have different rates of aquation which could account for the reduced clinical activity of carboplatin requiring higher doses to be administered to patients for the drug to be as effective as cisplatin (Ho et al., 2016).

This study used cultured prepubertal ovaries and testes to compare the gonadotoxicity of carboplatin and cisplatin. *In vitro* systems are widely used to test and compare different drugs in reproductive toxicology (Stefansdottir et al., 2014), and the methods used here are well-established models for culturing immature mouse gonadal tissue, supporting the health and physiological development of control cultured tissue (see for example Morgan et al., 2013; Smart et al., 2018). Whereas whole ovaries can remain healthy when cultured intact, the size of the testes results in large areas of necrosis if cultured whole, and so testicular tissue was dissected into small fragments prior to culture as carried out previously by ourselves and others (see for example Sato et al., 2011; Lopes et al., 2016; Smart et al., 2018). To compare the two drugs in both the ovary and the testis, a dose response for each drug was first obtained in order to determine a lowest dose that had no effect on that tissue and a highest dose that induced maximal damage, resulting in few, if any, healthy follicles in the ovary or surviving germ cells in the testis. The resulting dose response concentrations fall within clinical patient serum levels for each drug, with tissues exposed to higher concentrations of carboplatin than cisplatin, reflecting the relative dose administered to patients (Ruggiero et al., 2013).

Methods

Animals

Experiments were approved by the University of Edinburgh's Local Ethical Review Committee and carried out in accordance with UK Home Office regulations. CD1 mice were housed in an approved animal facility and kept in a 14-hr light: 10-hr dark cycle, with food and drink provided *ad libitum*. Animals were sacrificed through cervical dislocation.

Chemotherapy drugs

Cisplatin and carboplatin (Millipore, UK) were purchased in powder form, made up in distilled water to stock solutions of 1 mg/ml cisplatin and 10 mg/ml carboplatin. The chemotherapy drugs were added to the culture medium to produce final concentrations that resulted in a dose response for each tissue that ranged from a lowest dose that had no significant effect to a highest dose that affected the health (ovary) or number (testis) of the vast majority of germ cells. The dose response was based upon the health/number of the follicles/germ cells at the end of the culture period. For the ovary, concentrations of 0.5, 1 and 5 μg/ml cisplatin and 1, 5 and 10 μg/ml carboplatin were added; for the testis, lower concentrations of 0.01, 0.05 and 0.1 μg/ml cisplatin and 0.1, 0.5 and 1 μg/ml carboplatin were sufficient to produce the required dose response: all doses used were broadly within the range that has been found in patient serum, as detailed below.

Culture systems

Prepubertal mouse ovary and testis culture systems were performed as previously described and validated (Lopes *et al*., 2016; Smart *et al*., 2018), using hormone- and antibiotic-free culture conditions. Briefly, ovaries (postnatal day 4; pnd4) and testes (pnd5) were dissected and placed in Leibovitz L-15 medium (Invitrogen, UK) supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma, UK) prewarmed to 37°C.

Ovary culture

For each experimental run, ovaries were dissected from all females within a litter, with the ovaries then randomly distributed across treatment groups. Non-gonadal tissues were removed, and each ovary was cultured upon a floating polycarbonate membrane (Whatman Nuclepore Polycarbonate Membrane, Camlab Ltd, Cambridge, UK) in a 24-well plate (Greiner Bio-One, Stonehouse, UK) containing 1 ml α-minimum essential media (α-MEM, Invitrogen, UK) supplemented with 3 mg/ml fatty acid free BSA (Sigma, UK) and incubated at 37°C with 5% CO_2 (Day 1). Following 24 h of chemotherapy exposure (Day 2), tissues were removed and placed in a drug-free medium until the end of culture (Days 3–6), with media changes every 2/3 days. Vehicle control cultures containing drug-free media supplemented with water in place of drugs were maintained throughout the culture period. Tissues were fixed in Bouin's (Sigma, UK) for morphological analysis. For all groups, six ovaries were analysed ($n = 6$), obtained from at least four separate litters.

Testis culture

For each culture experiment, testes were obtained from two or three pups from the same litter. Non-gonadal tissues and surrounding tunica were removed, and testes were dissected into roughly 0.1-mm^3 fragments using a blade (Altomed, UK), with the testicular fragments then randomly distributed across treatment groups. Each fragment was cultured on a floating membrane within a 24-well plate containing 1 ml α-MEM media supplemented with 10% knockout serum replacement (Invitrogen, UK). Tissues were cultured at 37°C in 5% CO_2. After a settling in period of 24 h (Day 1), tissues were exposed to drugs for 24 h (Day 2), after which the floating membranes were moved into drug-free media (Day 3). To determine the proliferative capacity of tissues, 15 μg/ml of bromodeoxyuridine (BrdU; Sigma, UK) was added for the final 24 h of culture (Day 4). Vehicle control cultures containing drug-free media supplemented with water were maintained throughout the culture period. At end of the culture period, tissues were fixed in 10% neutral buffered formalin (NBF; Sigma, UK) for immunohistochemistry. For all groups, six fragments were analysed ($n = 6$), obtained from at least three separate litters.

Morphological evaluation of ovary

Fixed ovaries were embedded in paraffin and 5-μm sections cut. To evaluate morphology, follicle number, class and health were determined as in Morgan *et al.* (2013). Every sixth section was analysed, with total follicle counts estimated using the Abercrombie formula (Abercrombie, 1946). Tissues were stained with haematoxylin and eosin (H&E); images were taken with a DMLB Leica microscope (Leica Microsystems Ltd, UK) and analysed using ImageJ software (JAVA) with the assessor blind to experimental conditions. Follicles were included in the count only where germinal vesicles were visible. Follicle stage and health were determined based on appearance of granulosa cells and oocytes as described in Morgan *et al.* (2013). Follicles were classed as either primordial, transitional or primary, with overall health determined using standard morphological criteria as either unhealthy due to damage to the oocytes (Fig. 2 Aii, white arrowhead), to the granulosa cells (Fig. 2 Aiii, black arrowhead) or to both cell types. Oocyte damage was characterised based on the appearance of shrunken, eosinophilic cytoplasm or condensed nuclear chromatin, while granulosa cell damage was evidenced by the condensed chromatin or irregular overall shape of at least one granulosa cell, as detailed in previous work by ourselves and others (see for example Springer *et al.*, 1996; Muruvi *et al.*, 2005; Morgan *et al.*, 2013; Lopes *et al.*, 2014, 2016).

Immunohistochemistry of testis

Fixed testis fragments were embedded in paraffin and serially sectioned at 5 μm. Every fifth section was immunostained. In brief, slides were dewaxed, rehydrated and subjected to antigen retrieval with citrate buffer (10 mM sodium citrate, pH 6, Fisher Chemical, UK). Slides were blocked in 20% goat serum in PBS (Thermo Fisher Ltd, UK) with 0.1% Triton X-100 (PBST; Sigma, UK) and 5% BSA for 1 h. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 h at room temperature in 10% goat serum, PBST and 5% BSA; see Table I for all antibody details. Slides were counterstained with DAPI (Invitrogen, UK) and mounted with Vectashield mounting medium (Vector Laboratories, USA). Images were taken (Leica DM4400B microscope on a DFC360FX camera) and analysed using ImageJ (JAVA) with the assessor blind to experimental conditions. The

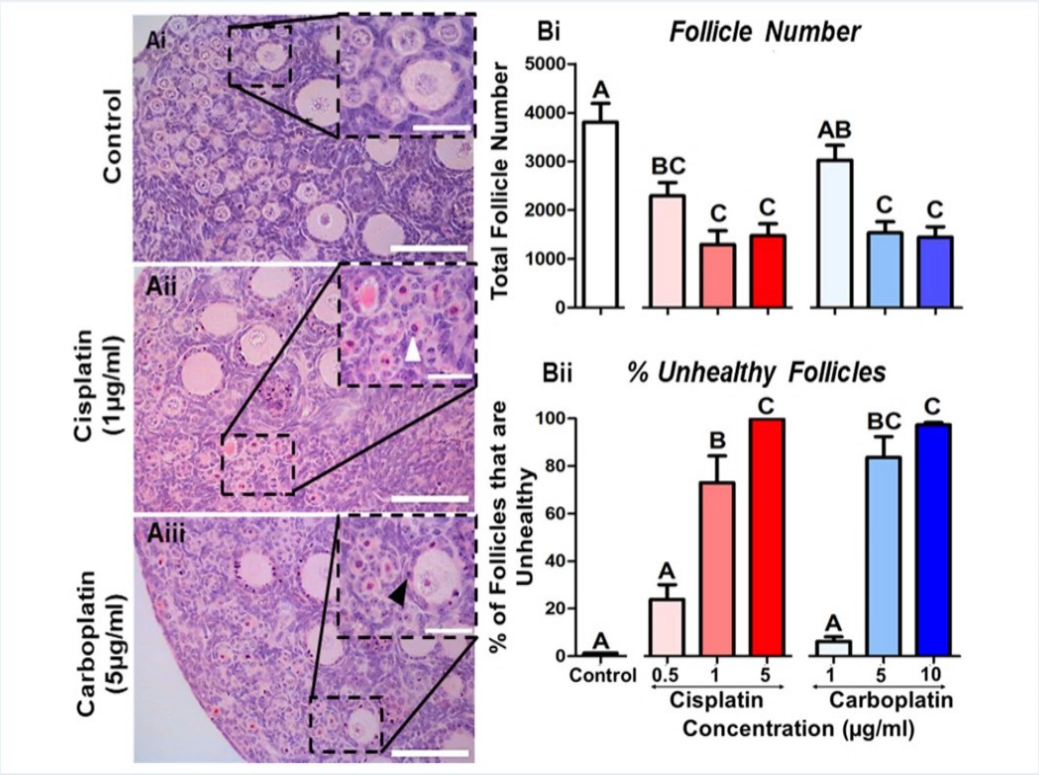


Figure 2 Follicle number and health are reduced following cisplatin or carboplatin treatment. (A) Representative histological sections of (Ai) control, (Aii) cisplatin (1 µg/ml) and (Aiii) carboplatin (5 µg/ml) exposed tissue; insets show higher magnification of framed areas. White arrowhead shows example of a damaged oocyte; black arrowhead shows example of a damaged granulosa cell; scale bars represent 50 µm, or 20 µm for insets. (**B**) Histograms show (Bi) total follicle number and (Bii) the percentage of unhealthy follicles following exposure to cisplatin (red) or carboplatin (blue); $n = 6$ for all experimental conditions. Data are mean + SEM; columns without letters in common are statistically significantly different to each other ($P < 0.05$).

density of total proliferating germ cells (MVH-positive/BrdU-positive), non-proliferating germ cells (MVH-positive/BrdU-negative) and proliferating Sertoli cells (SOX9-positive/BrdU-positive) were determined through manual counting per area of seminiferous tubule. Area of fluorophore expression for Sertoli cells (SOX9-positive) per area of seminiferous tubules was used to determine density of Sertoli cells as described in Smart *et al.* (2018).

Statistical analysis

Statistical analysis was performed using Minitab statistical software programme version 17 (Minitab Inc., State College, PA, USA), and graphs were created using GraphPad Prism software version 5 (GraphPad Software, Inc., CA, USA). A one-way ANOVA with general linear mixed effect model was used to determine statistical significance of treatment, with effect of culture run taken into account; results were deemed statistically significant at $P < 0.05$. Tukey pairwise comparison test determined statistical significance between the experimental conditions. Histograms of results are represented as mean + SEM. Significance between groups is symbolised on graphs by letters, with different letters indicating a statistical significance difference with individual P values of $P < 0.05$.

Results

Cisplatin or carboplatin exposure reduces the number and health of follicles in the prepubertal ovary

In order to assess the effects of cisplatin or carboplatin treatment, ovaries were examined after exposure *in vitro* to a range of concentrations that gave a dose response (cisplatin—0.5, 1 and 5 µg/ml; carboplatin—1, 5 and 10 µg/ml; Fig. 2A). Total follicle number was reduced compared to controls following exposure to all concentrations of cisplatin, with a reduction from 0.5 µg/ml (40%; $P = 0.007$), 1 µg/ml (66%; $P = 0.001$) and 5 µg/ml (61%; $P = 0.001$) and from 5 µg/ml (60%; $P = 0.001$) and 10 µg/ml (62%; $P = 0.001$) of carboplatin (Fig. 2 Bi). The

Table I Primary and secondary antibodies for double immunohistochemistry.

Antigen 1	*Dilution*	*Secondary Antibody for Antigen 1*	*Antigen 2*	*Dilution*	*Secondary Antibody for Antigen 2*
Mvh (ab27591)	1:200	GAMou-488	BrdU (ab6326)	1:500	GARat-568
Sox9 (ab5535)	1:500	GARab-488	BrdU (ab6326)	1:500	GARat-568

Primary antibodies (antigen 1 and 2) purchased from Abcam, UK, and Secondary antibodies purchased from Invitrogen, UK.

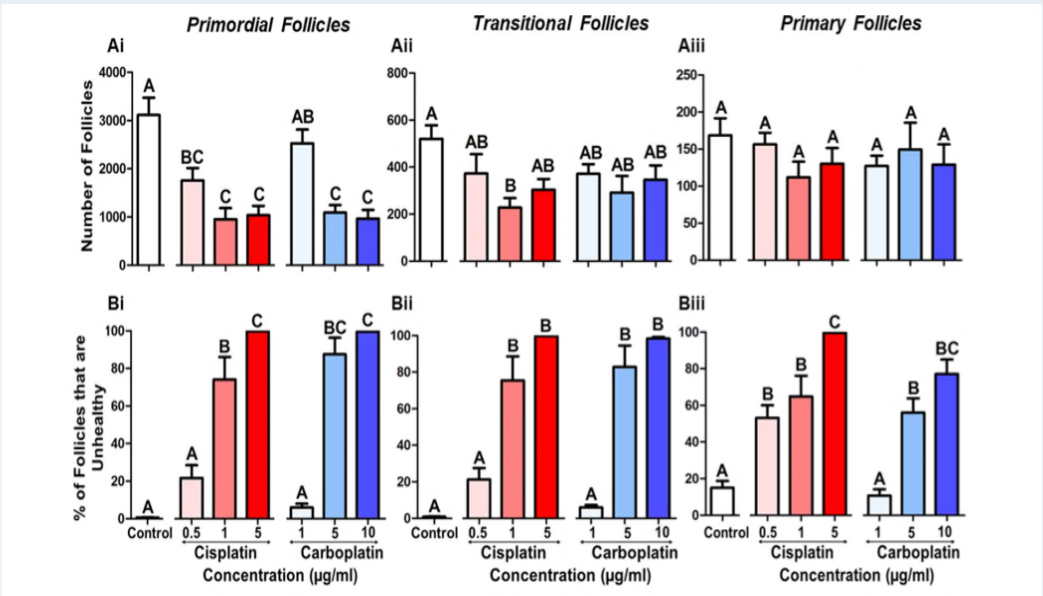


Figure 3 Cisplatin or carboplatin treatment reduces the number of primordial follicles while damaging the health of all follicles. **(A)** number and (**B**) percentage of follicles classified as unhealthy for (i) primordial, (ii) transitional or (iii) primary follicles following cisplatin (red) or carboplatin (blue) treatment, $n = 6$ for all experimental conditions. Data are mean + SEM; columns without letters in common are statistically significantly different to each other ($P < 0.05$).

percentage of unhealthy follicles increased in a dose-dependent manner when exposed to at least 1 µg/ml of cisplatin (73 + 11% unhealthy; $P = 0.001$) and 5 µg/ml carboplatin (84 + 9% unhealthy; $P = 0.001$) in comparison to the control (Fig. 2 Bii). At the highest concentrations, almost all follicles were unhealthy at 5 µg/ml cisplatin (100% unhealthy; $P = 0.001$) and 10 µg/ml carboplatin (97 + 1% unhealthy; $P = 0.001$).

Cisplatin or carboplatin targets follicles independent of stage

Since the number and health of the follicles were reduced following treatment with either drug, the follicles were further classified as primordial, transitional or primary to determine if a particular follicle class was targeted. Both cisplatin and carboplatin reduced the number of primordial follicles in comparison to the control from 0.5 µg/ml cisplatin (44%; $P = 0.004$) and 5 µg/ml carboplatin (65%; $P = 0.001$) (Fig. 3 Ai). There was little effect of cisplatin or carboplatin treatment on the number of growing transitional or primary follicles, although a trend towards a decrease in transitional follicles was noted, with a significant reduction in number at 1 µg/ml (56%; $P = 0.029$) of cisplatin only (Fig. 3 Aii,iii). When follicle health rather than number was examined, the percentage of unhealthy follicles increased for all classes in a dose-dependent manner. Damage to the primordial follicles was evident from 1 µg/ml cisplatin (74 + 12% unhealthy; $P = 0.001$) and 5 µg/ml carboplatin (87 + 9% unhealthy; $P = 0.001$) (Fig. 3 Bi). A significant number of unhealthy transitional follicles was observed from 1 µg/ml cisplatin (75 + 13% unhealthy; $P = 0.001$) and 5 µg/ml carboplatin (83 + 12% unhealthy; $P = 0.001$) (Fig. 3 Bii). For the primary follicles, damage was seen from 0.5 µg/ml cisplatin (53 + 7% unhealthy; $P = 0.005$) and 5 µg/ml carboplatin (56 + 8% unhealthy; $P = 0.002$) (Fig. 3 Biii).

Granulosa cells of primary follicles are damaged by cisplatin or carboplatin exposure

The health of each primary follicle was assessed depending upon whether the oocyte and/or granulosa cells were damaged following

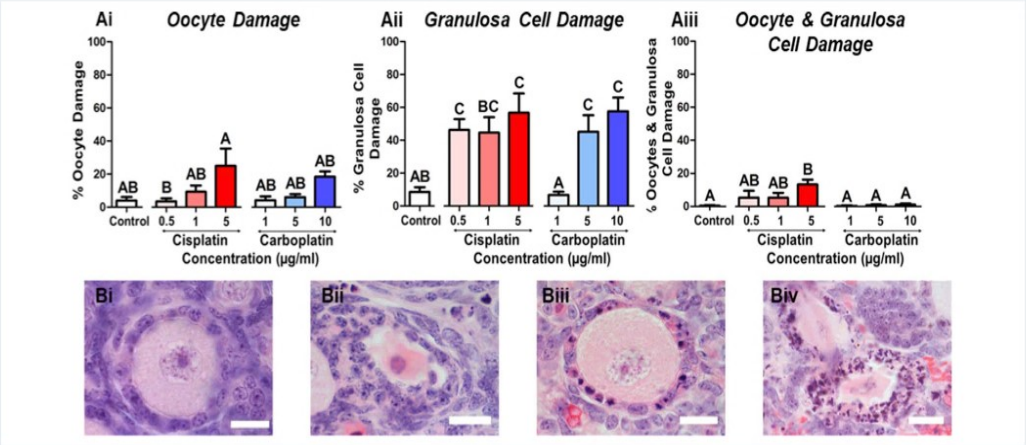


Figure 4 Granulosa cells of the primary follicles are damaged by cisplatin or carboplatin. Percentage of primary follicles classed as unhealthy due to (Ai) oocyte, (Aii) granulosa cell or (Aiii) combined (oocyte and granulosa cell) damage following cisplatin (red) or carboplatin (blue) treatment, $n = 6$ for all experimental conditions. Data are mean + SEM; columns without letters in common are statistically significantly different to each other ($P < 0.05$). Representative images show (Bi) healthy primary follicle (Bii) primary follicle with damaged oocyte, (Biii) primary follicle with damaged granulosa cells and (Biv) primary follicles with combined (oocyte and granulosa cell) damage. Scale bar = 10 µm.

cisplatin or carboplatin exposure (Fig. 4). This analysis was not carried out for primordial or transitional follicles due to the difficulty of properly assessing the health of flattened granulosa cells. In primary follicles, either cisplatin or carboplatin treatment damaged the granulosa cells in comparison to control. These effects were seen from 0.5 µg/ml cisplatin (46 + 7% unhealthy; $P = 0.037$) and from 5 µg/ml carboplatin (45 + 10% unhealthy; $P = 0.049$) (Fig. 4 Aii).

Cisplatin or carboplatin exposure reduces the proliferating germ cell population in the prepubertal testis

To determine the impact of cisplatin or carboplatin treatment on the prepubertal testis, testicular fragments were exposed to cisplatin (0.01, 0.05 and 0.1 µg/ml) or carboplatin (0.1, 0.5 and 1 µg/ml) *in vitro* (Fig. 5 A). Germ cells (MVH-positive) were counted and classified as proliferating (MVH-positive/BrdU-positive) or non-proliferating (MVH-positive/BrdU-negative). The density of the total germ cell population was significantly reduced in a dose-dependent manner evident from 0.05 µg/ml cisplatin (71% decrease; $P = 0.001$) and 0.5 µg/ml carboplatin (75% decrease; $P = 0.001$) (Fig. 5 Bi). A low density of germ cells remained after treatment at the highest concentrations of both drugs in comparison to control with 15% of germ cells remaining after cisplatin (0.1 µg/ml; $P = 0.001$) and 12% after carboplatin (1 µg/ml; $P = 0.001$) treatment. Cisplatin and carboplatin both affected the proliferating germ cell population (MVH-positive/BrdU-positive), with the density significantly decreased in a dose-dependent manner following exposure to at least 0.05 µg/ml cisplatin (73% decrease; $P = 0.001$) and 0.5 µg/ml carboplatin (75% decrease; $P = 0.001$) (Fig. 5 Bii). A small population of non-proliferating germ cells (MVH-positive/BrdU-negative) was observed in the control tissue, with a similar density (~400 + 30 cells per mm^2 of tubule) of these cells maintained across all experimental groups.

Sertoli cell density is unaffected by either cisplatin or carboplatin exposure

To assess whether the damaging effect of cisplatin or carboplatin exposure on germ cell density was also seen on the somatic cells present within the seminiferous tubules, we determined the overall density of Sertoli cells (SOX9-positive) (Fig. 6 A). This was unaffected by cisplatin or carboplatin exposure ($P = 0.641$) (Fig. 6 Bi). Similarly, the proliferative capacity of these cells (SOX9-positive/BrdU-positive) were also not altered by exposure to either drug ($P = 0.283$) (Fig. 6 Bii).

Discussion

Prepubertal mouse ovaries and testes were exposed to a range of concentrations of either cisplatin or carboplatin in culture, within patient relevant concentrations; both drugs induced a dose-dependent response. In order to achieve a similar clinical efficacy, carboplatin must be administered at concentrations 5–15 times higher than cisplatin (Hongo *et al.*, 1994; Dasari and Tchounwou, 2014). Here, results show that for ovarian tissue, two to five times carboplatin concentrations (1, 5 and 10 µg/ml) in comparison to cisplatin (0.5, 1 and 5 µg/ml) induced equivalent levels of damage to the follicles, which resulted in significant effects at or above 1 µg/ml cisplatin and 5 µg/ml carboplatin in terms of health. For the testicular tissue, 10 times carboplatin doses (0.1, 0.5 and 1 µg/ml) were required to induce a similar reduction in germ cell density compared to cisplatin doses (0.01, 0.05 and 0.1 µg/ml), resulting in significant effects at or above 0.05 µg/ml cisplatin and 0.5 µg/ml carboplatin.

This is the first study to compare the effects of cisplatin or carboplatin exposure on the prepubertal gonad in females and males. Several clinical studies in adults have compared the efficacy, survival and toxicities of these drugs in treating a range of cancers where cisplatin has, in general, better survival rates and efficacy while carboplatin has a

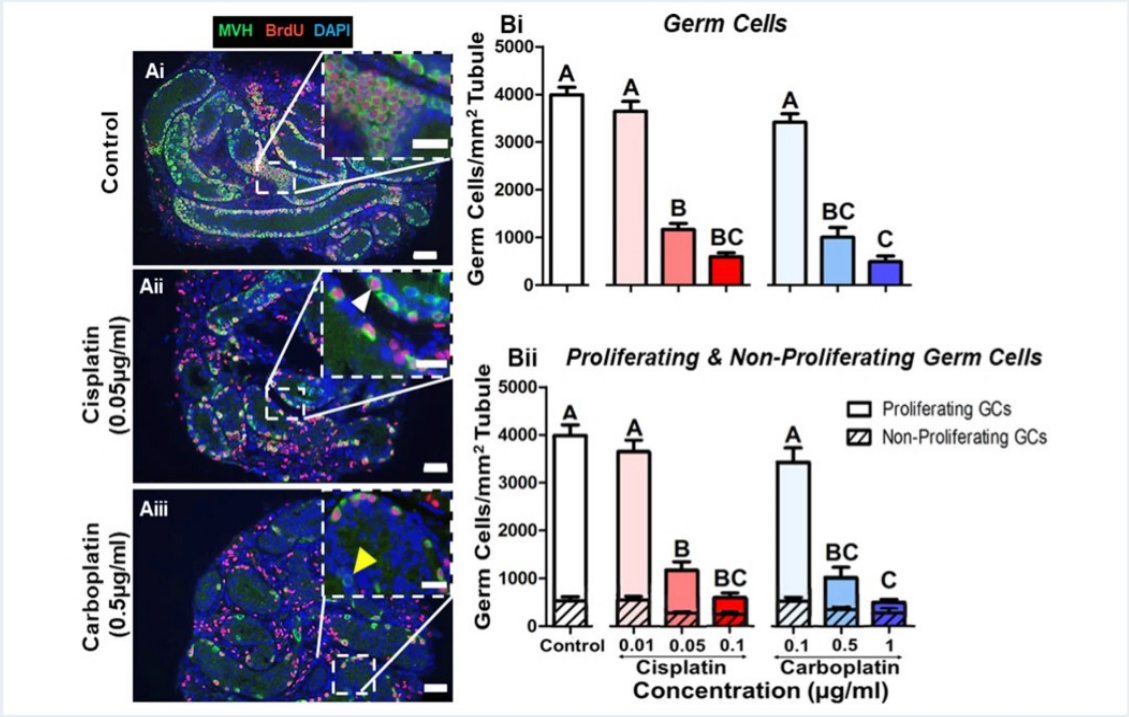


Figure 5 Cisplatin or carboplatin treatment results in a significant loss of the proliferating germ cells. (**A**) Representative images of histological sections from (Ai) control, (Aii) cisplatin (0.05 µg/ml) and (Aiii) carboplatin (0.5 µg/ml) treated testis, showing a reduction in the density of proliferating germ cells (MVH-positive, green; BrdU-positive, red). Insets show higher magnification of framed areas. White arrowhead shows example of a proliferating germ cell (MVH-positive/BrdU-positive); yellow arrowhead shows example of a non-proliferating germ cell (MVH-positive/BrdU-negative). Scale bars represent 50 µm, or 25 µm for insets. (**B**) Density of germ cells per seminiferous tubule area (mm^2) following cisplatin (red) or carboplatin (blue) treatment. Data shows the density of the (Bi) total, (Bii: open bars) proliferating and (Bii: hatched bar) non-proliferating germ cells; $n = 6$ for all experimental conditions. Data are mean + SEM; columns without letters in common are statistically significantly different to each other ($P < 0.05$). For (Bii), letters refer to proliferating germ cells (open bars) only, with no statistically significant difference between groups for the non-proliferating germ cells (hatched bars).

better toxicity profile; however, this is not the case for all malignancies (reviewed in Ho *et al.*, 2016). For paediatric patients, carboplatin has been found to be as effective as cisplatin in treating germ cell tumours with the added benefit of a reduced toxicity profile (Ruggiero *et al.*, 2013; Frazier *et al.*, 2018; Shah *et al.*, 2018). The toxicity of these platinum alkylating-like drugs is mainly attributed to the formation of DNA adducts, resulting in cross-linkage and subsequent DNA damage: whether there is any underlying mechanism that may account for the observed differences in toxicity prolife of these two drugs is, however, not yet clear (reviewed in Siddik, 2003; Dasari and Tchounwou, 2014). One study in cancer cell lines has identified a potential difference between the two drugs, with cisplatin producing higher levels of oxidative stress than carboplatin at patient-equivalent concentrations (Marullo *et al.*, 2013); it might be of interest to explore the oxidative stress response to cisplatin and carboplatin in the gonads in future studies. Our study compares the effects of these drugs on prepubertal gonadal tissues and found, relative to the concentrations of each drug in patient serum, gonadotoxicity resulting from carboplatin exposure was at least as bad as that resulting from cisplatin exposure (patient serum levels: cisplatin—0.05-4 µg/ml; Crom *et al.* 1981; Dominici *et al.* 1989; Peng *et al.* 1997; Erdlenbruch *et al.* 2001 and carboplatin—0.5–90 µg/ml; Madden *et al.*, 1992; Murry *et al.*, 1993; Newell *et al.*, 1993; Riccardi *et al.*, 1994; Clerico *et al.*, 2006; Veal *et al.*, 2007). This is of particular importance, as carboplatin as a second-generation drug of the platinum alkylating-like class was developed as an alternative to cisplatin in part due to reduced nephrotoxicity, ototoxicity and emesis for paediatric patients (reviewed in Ruggiero *et al.*, 2013). Overall, carboplatin doses of two to five times for the ovary and 10 times for the testis were found to be as damaging as equivalent cisplatin concentrations. The same conclusion was also reached in an *in vivo* study of adult mice based on the morphology of the testis, where 10 times carboplatin was found to be as gonadotoxic as cisplatin (Köpf-Maier, 1992). Reduced spermatogenesis and altered structure of the seminiferous tubules were observed, as well as decreased integrity of Sertoli cells disrupting tight junction contact, a factor that was not investigated in our study. Another such study of adult males found carboplatin to be less toxic than cisplatin with regard to steroidogenesis (Azouri *et al.*, 1989): our study did not investigate the impact on steroidogenesis. Our data from this *in vitro*, animal model study suggests that replacing cisplatin with carboplatin in treatment regimens will not

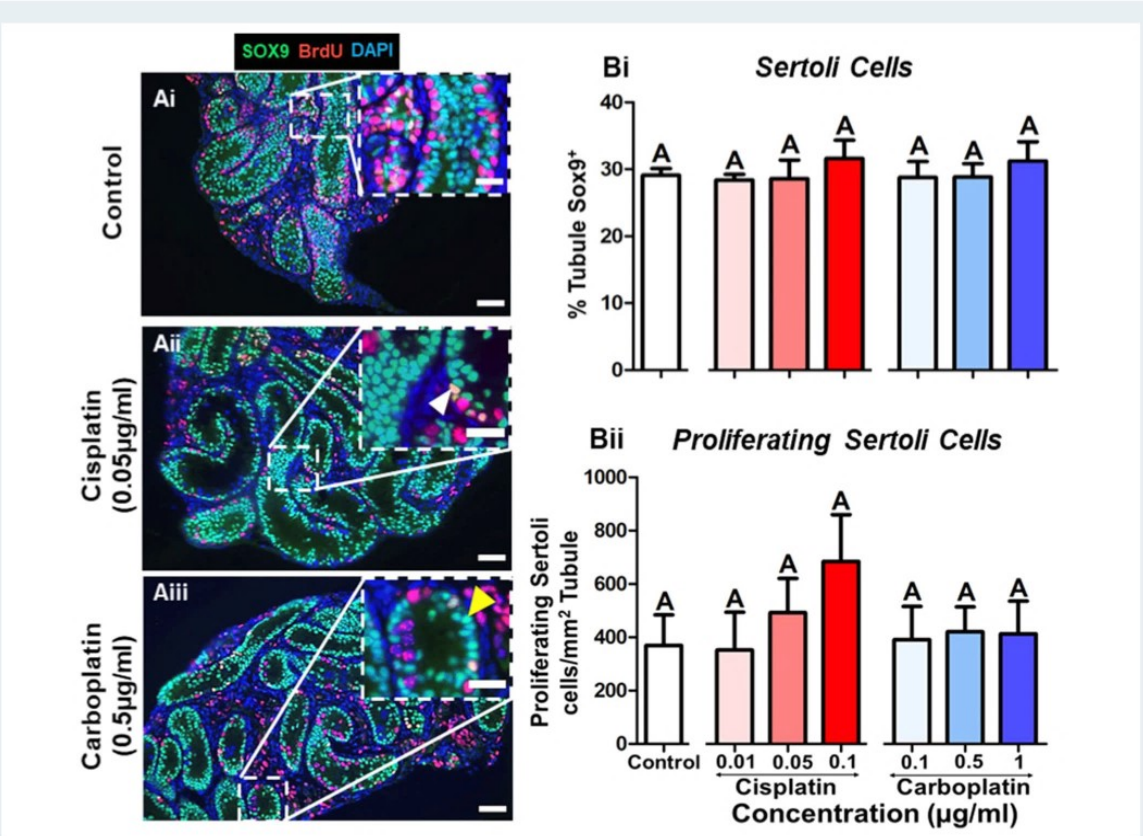


Figure 6 Density and proliferation of Sertoli cells are not affected by cisplatin or carboplatin. (A) Representative images of (Ai) control, (Aii) cisplatin (0.05 µg/ml) and (Aiii) carboplatin (0.5 µg/ml) treated tissues showing no change in density of Sertoli cells (SOX9-positive; green) or in proliferating Sertoli cells (SOX9-positive/BrdU-positive; orange). Insets show higher magnification of framed areas. White arrowhead shows example of a proliferating Sertoli cell; yellow arrowhead shows example of a non-proliferating Sertoli cell; scale bars represent 50 µm, or 25 µm for insets. (**B**) Data shows (Bi) density of Sertoli cells measured by the % area of the seminiferous tubules expressing Sox9, following cisplatin (red) or carboplatin (blue) treatment as well as (Bii) number of manually counted proliferating Sertoli cells. $n = 6$ for all experimental conditions. Data are mean + SEM; columns with different letters are statistically significantly different to each other ($P < 0.05$).

prevent or reduce chemotherapy-induced gonadotoxicity. Based on our short-term study, carboplatin may be even more damaging to the ovary than cisplatin, since only two to five times the carboplatin concentration induced equivalent damage as the cisplatin, whereas patients can receive up to 15 times the dose. It will be important for further studies to focus on clinical paediatric patients, to determine whether the same conclusions can be drawn on future fertility as from this short-term animal study.

For both cisplatin and carboplatin, higher doses were required to observe significant levels of germ cell/follicle damage/loss to the ovarian tissue in comparison to the testes, suggesting the testis may be more sensitive to the platinum alkylating-like agents. This has previously been noted for other chemotherapy drugs including irinotecan (Lopes *et al.*, 2016), although that could, in part at least, be due to differences in the culture conditions of ovarian and testicular tissue. It is also important to bear in mind that, whereas there would be the potential for even a few surviving spermatogonial stem cells to (partially) repopulate the testis after treatment, any loss of oocytes would be irreplaceable. Nonetheless, in agreement with our current and previous (Lopes *et al.*, 2016) findings that the testis may be more sensitive to chemotherapy treatment than the ovary, a study following survivors of childhood cancer that had been treated with alkylating agents found that the effects on male fertility were more pronounced than those on female fertility (Chow *et al.*, 2016); the decrease in the percentage of male survivors siring a live birth compared to their siblings was greater than the decrease in the percentage of female survivors having live births compared to their siblings. Additional studies are required to determine the functional deficits following cisplatin or carboplatin treatment and the long-term impact on recovery and subsequent effects on fertility.

Ovarian toxicity as a result of chemotherapy treatment can lead to infertility and premature ovarian insufficiency (POI) due to direct damage to the ovary depleting the ovarian reserve of primordial follicles (reviewed in Ben-Aharon and Shalgi, 2012; Morgan *et al.*, 2012; Bedoschi *et al.*, 2016; Spears *et al.*, 2019). Primordial follicles form before birth when the germ cells leave the mitotic phase, enter meiosis, arrest at prophase I and become surrounded by pregranulosa cells.

These primordial follicles represent a female's reproductive potential and are activated into growing follicles throughout the reproductive lifespan of the female (McGee and Hsueh, 2000). Loss of primordial follicles, through for example chemotherapy treatment, can lead to POI and early menopause (Wallace *et al.*, 2005). Our study shows that cisplatin and carboplatin exposure leads to a reduction in the number of primordial follicles aligning with previous studies on cisplatin (Meirow, 2000; Gonfloni *et al.*, 2009; Morgan *et al.*, 2013). All follicles, including those at the primordial state, were damaged in our study, showing that direct effects of the drugs on the primordial follicles is at least part of the reason for the primordial follicle loss. Overall, primordial follicle loss may be a result of either direct damage to the primordial follicles and/or accelerated activation of primordial follicles into the growth phase as a result of the drugs depleting the growing follicular pool that usually inhibits activation (Meirow *et al.*, 2010; Findlay *et al.*, 2015). In the literature, the underlying mechanism for primordial follicle loss following exposure to platinum alkylating-like agents is still debated, with some studies showing direct damage to the primordial follicles (Nguyen *et al.*, 2019) and others suggesting that accelerated activation contributes to loss of primordial follicles following cisplatin treatment (Morgan *et al.*, 2013; Chang *et al.*, 2015).

The specific cell type within primary ovarian follicles that was affected by cisplatin or carboplatin treatment was further investigated, to determine whether the oocyte and/or granulosa cells were targeted. The oocyte and surrounding granulosa cells are co-dependent upon each other for oocyte maturation and folliculogenesis, therefore damage to one cell type will ultimately result in death of the latter, but it is also important to know which cell type is affected first if protective treatments are to be developed. Our results show that for primary follicles, both cisplatin and carboplatin primarily damage granulosa cells. Previous studies have focused on the oocyte or have combined primary follicles with transitional follicles to represent the growing follicular pool (Gonfloni *et al.*, 2009; Morgan *et al.*, 2013). These studies have shown effects of cisplatin on the oocyte, as a result of DNA damage, accumulation of Tap63 and activation of apoptotic pathways (Gonfloni *et al.*, 2009; Kerr *et al.*, 2012; Kim *et al.*, 2013, 2019; Morgan *et al.*, 2013; Rossi *et al.*, 2017; Nguyen *et al.*, 2018, 2019). These studies came to the conclusion that cisplatin targeted the oocyte, whereas our study found that for primary follicles, mainly granulosa cells were damaged following cisplatin treatment. The difference between these findings and the results of the present study is not clear, but could in part, be due to differences in mouse line (here CD1 line) or age of animal (here, pnd 4). The mechanism underlying granulosa cell damage following treatment with platinum containing alkylating-like agents has yet to be investigated fully.

For the testis, there is much less known about the effects of chemotherapy treatment on the prepubertal gonad than that of the adult. The paucity of prepubertal studies could possibly be due to limited animal model systems to investigate prepubertal testis development and/or to the fact that the prepubertal testis was once considered relatively inactive and therefore thought to be protected from any chemotherapy-induced damage (Rivkees and Crawford, 1988; Rey, 1999). The testis undergoes important developmental processes during prepuberty, including germ cell proliferation and Sertoli cell functional maturation, any of which could be affected by chemotherapy drugs (Chemes, 2001; Allen *et al.*, 2018). The present study shows that both cisplatin and carboplatin reduce the population of proliferating germ cells in the prepubertal testis. The proliferative capacity was analysed during a 24-h period starting 24 h after drug exposure had finished; therefore, the loss of proliferating cells could either be a result of the drugs targeting and killing the proliferating cells or a consequence of the drugs preventing subsequent germ cell proliferation. The underlying mechanism of action of the platinum alkylating-like agents has been studied following cisplatin treatment and indicates that cisplatin induces DNA damage and activates apoptotic pathways as shown through increased expression of cleaved caspases 3, TUNEL-positive cells and γH2AX expression (Lirdi *et al.*, 2008; Favareto *et al.*, 2011; Smart *et al.*, 2018). The germ cell population at this stage of development is predominantly composed of undifferentiated type A spermatogonia (spermatogonial stem cell; SSC) as well as differentiating spermatogonia type B (de Rooij and Russell, 2000; Ehmcke *et al.*, 2006). Further investigations should therefore focus on the specific germ cell types targeted, since our study used a generic marker for germ cells, MVH. Research is focusing on SSCs, since maintenance of this population is crucial for later fertility (Zohni *et al.*, 2012; Zohni *et al.*, 2012). SSCs are vulnerable to cisplatin treatment, with survival of isolated SSCs decreased in culture (Marcon *et al.*, 2010; Liu *et al.*, 2014; Shabani *et al.*, 2016) and PLZF-positive germ cells (SSCs) reduced in cultured testis tissue following cisplatin exposure (Smart *et al.*, 2018).

The density of the Sertoli cell population was unaffected by either cisplatin or carboplatin exposure as previously shown in Smart *et al.* (2018). However, there is the potential that functional maturation of these cells could be affected, resulting in Sertoli cells that are not capable of fully supporting germ cells through spermatogenesis in the adult (Chemes, 2001; Sharpe *et al.*, 2003). Indeed, previous studies have found a decrease in Sertoli cell viability (Aslani *et al.*, 2017) following treatment as well as functional deficits through reduced androgen binding protein or transferrin production (Nambu *et al.*, 1995; Favareto *et al.*, 2011). Here, additional studies would be required to investigate the maturation of these cells, through for example analysis of anti-Müllerian hormone and inhibin B (Dere *et al.*, 2013). In addition to the Sertoli cells, other somatic cells within the testis, including the endocrine Leydig cells and peritubular myoid cells, could be affected by chemotherapy treatment but have yet to be fully investigated. Initial studies using a mouse model found no impact of cisplatin on the density of the Leydig cell population (Smart *et al.*, 2018); however, there have yet to be any studies focusing on steroidogenesis or testosterone production following prepubertal exposure to either cisplatin or carboplatin.

In summary, working within the range of drug concentrations found in patient serum, results here show that clinically relevant concentrations of cisplatin and carboplatin have similar gonadotoxic effects on the prepubertal mouse ovary and testis. Both platinum-alkylating agents reduced the number and health of ovarian follicles. In the testis, either drug reduced the density of the proliferating germ cell population, while no effect on the density of Sertoli cells was seen. Since both drugs had an impact on gonadal tissues within clinically relevant concentrations, we conclude from this *in vitro* animal model study that replacing cisplatin with carboplatin in treatment protocols may not result in any additional protection of the gonadal tissues from the damaging effects of the drugs. Further clinical studies are required before any recommendations for paediatric patients can be made.

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Authors' roles

C.M.A.: participated in the experimental design of the study, led experiments, analysed data, prepared figures and wrote the manuscript; F.L.: participated in the experimental design of the study and in experiments and commented on earlier versions of the manuscript; R.T.M.: participated in the experimental design of the study and commented on earlier versions of the manuscript; N.S.: conceived, designed and coordinated the study and helped draft the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

No authors have any conflict of interest to declare.

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3.3 Discussion

3.3.1 Overview of results

The development of newer generations of chemotherapy drugs and the use of treatment protocols that include less 'toxic' chemotherapy agents are strategies that can limit chemotherapy-induced damage to healthy tissues. In relation to prepubertal reproductive tissues, the gonadotoxicity classification of chemotherapy agents are debated since there is a lack of data specifically exploring this in prepubertal patients or animal models (see 1.4 for human data and 1.5 for animal data). The results from this investigation show that, of the platinum alkylating-like drug class, the first-generation drug cisplatin and second-generation drug carboplatin have similar gonadotoxicity profiles in prepubertal mouse ovaries and testes. The prepubertal gonads were cultured for four-six days and exposed to the chemotherapy drugs for 24hrs on the second day of culture. The two drugs were compared by examining the concentrations that gave a dose-response for each drug upon the reproductive tissues, with a 'low' dose that had little to no impact on follicle health/germ cell density, as well as a 'high' dose that had an effect on the majority of follicles/germ cells. Clinically, carboplatin is administered at 5-15 times the cisplatin dose to be as effective, which results in higher patient serum levels of carboplatin. This is reflected in patient serum levels of cisplatin (0.05-4µg/ml; Crom *et al.*, 1981; Dominici *et al.*, 1989; Peng *et al.*, 1997; Erdlenbruch *et al.*, 2001; Liston and Davis, 2017), compared to carboplatin (0.5-90µg/ml; Madden *et al.*, 1992; Murry *et al.*, 1993; Newell *et al.*, 1993; Riccardi *et al.*, 1994; Clerico *et al.*, 2006; Veal *et al.*, 2007; Datta *et al.*, 2013; Liston and Davis, 2017). In these cultures, similarly higher concentrations of carboplatin were required to obtain a dose-response. In the testis; ten times carboplatin doses were as gonadotoxic as cisplatin doses, whereas in the ovary; two-five times carboplatin induced equivalent damage as cisplatin. These doses are clinically relevant and show carboplatin to be as gonadotoxic as cisplatin to both the prepubertal ovary and testis.

3.3.2 Changes to methodology and approach to statistical analysis

These results were obtained during the first year of the PhD programme. Given that, further years of the PhD provided many opportunities to reflect upon the experimental design and statistical analysis, with changes implemented based on these reflections in further Chapters.

The testis fragments were cultured at 37^{o}C, the same as the ovaries, as had previously been used within our laboratory to successfully culture testis fragments, with the germ cell population and overall morphology preserved (Lopes *et al.*, 2016; Smart *et al.*, 2018). Research with long-term cultures of the testis, however, have shown the benefit of culturing at 34^{o}C: this is a more physiologically relevant temperature, an environmental aspect that germ cells and spermatogenesis are sensitive to (Gohbara *et al.*, 2010). Subsequent testis cultures described in remaining Chapters were therefore performed at 34^{o}C (see Chapter 2.1). In addition, the size of the testis fragments were later enlarged from 0.1mm^3 to 1mm^3 to increase the amount of tissue collected from these cultures for analysis. The final change made after the work described here was in relation to what constitutes the 'n' number. Within this study, the testis fragments for analysis were obtained from three cultures and therefore litters, with multiple fragments coming from the same culture run/litter to give a total sample size, or 'n' number, of six. To statistically analyse the data collected here, a general linear mixed effects model was used to take into account potential 'random effects'. In order words, this statistical model will take into account the fact that multiple fragments were obtained from the same litter, to try to counter any effects of pseudo-replication. Following on from this work, the experimental design was changed so that in future chapters, an 'n' of one is considered tissue derived from a single litter, with testis fragments from the same litter considered replicates (see Chapter 2, 2.9). That change was made to eliminate potential pseudo-replication and the 'random effects' of culture run, therefore further analysis did not include a general linear mixed effects model.

Following later analysis that involved fitting data to a sigmoidal dose-response curve (see 5.3.3), re-analysis of the data presented in this paper was performed. This was accomplished in Graphpad (see 5.2.4) by fitting a curve with nonlinear regression using a sigmoidal model/equation that is equivalent to a dose-response curve. This equation generates values for the EC50 dose i.e. concentration which induces a halfway response between the baseline and maximal response, which can be compared between the two drugs. The data is shown in Fig 3.1, with the dose-response curves for cisplatin and carboplatin superimposed upon each other for density of germ cells in the testis and total number of follicles in the ovary. When the EC50 doses were compared between the two drugs, it is clear that the EC50 dose for carboplatin (0.2µg/ml) is 10X the EC50 dose for cisplatin (0.02µg/ml), with the drugs producing remarkably similar dose-response curves, confirming my initial observations. For the ovary, the EC50 dose for carboplatin (1.3µg/ml) is 4.3X the EC50 dose of cisplatin (0.3µg/ml).

3.3.3 Confirmation of findings from in vitro human testis culture model

Conclusions from this paper were based on results obtained from cultured mouse prepubertal reproductive tissues. Before any clinical recommendations can be made, however, further research utilizing human tissues and/or clinical data is required to confirm these animal model findings. Further work performed within the laboratory of Prof Rod Mitchell has compared cisplatin and carboplatin utilizing cultured human fetal testis tissue (Tharmalingam MD, Matilionyte G, Wallace WHB, Stukenborg JB, Jahnukainen K, Oliver E, Goriely A, Lane S, Guo J, Cairns B, Jorgensen A, **Allen CM**, Lopes F, Anderson RA, Spears N and Mitchell RT 'Exposure to platinum-based chemotherapy causes differential loss of germ cell sub populations in human fetal and prepubertal testis'; Revised version of manuscript currently under review by BMC Medicine). The results confirm our findings whereby a similar loss of germ cells was observed 240hrs (10 days) post-exposure to 0.5µg/ml cisplatin or 5µg/ml carboplatin.

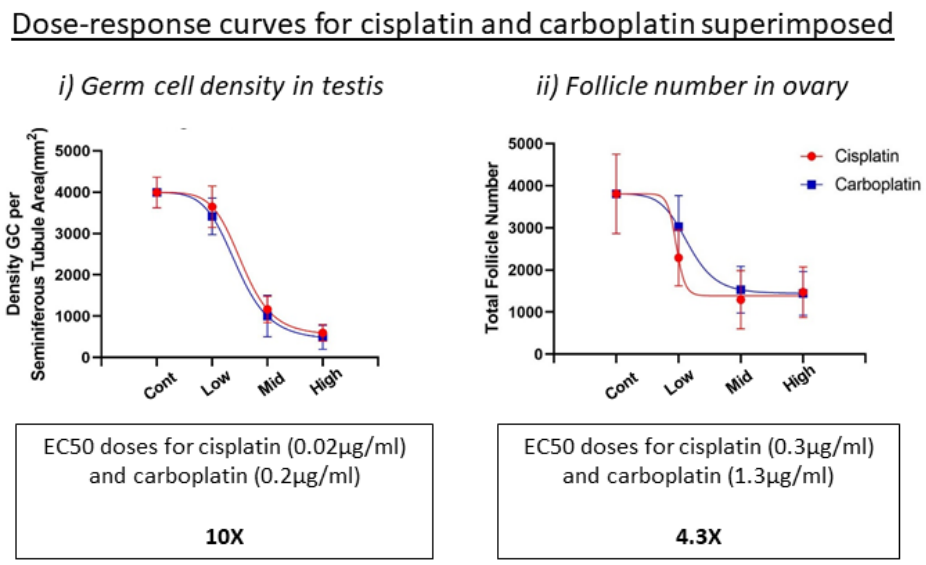


Figure 3.1: Dose-response curves for cisplatin- and carboplatin-induced damage to cultured prepubertal mouse gonads. The dose-response curves for cisplatin (red) or carboplatin (blue) treatment were superimposed to show the impact on the i) density of germ cells in the testis fragments and ii) total number of follicles in the ovary. For the testis, 'low' concentrations were 0.01µg/ml cisplatin and 0.1 µg/ml carboplatin, 'mid' concentrations 0.05 µg/ml cisplatin and 0.5 µg/ml carboplatin, high concentrations 0.1 µg/ml cisplatin and 1 µg/ml carboplatin. For the ovary, 'low' concentrations were 0.5µg/ml cisplatin and 1 µg/ml carboplatin, 'mid' concentrations 1µg/ml cisplatin and 5µg/ml carboplatin, high concentrations 5µg/ml cisplatin and 10 µg/ml carboplatin. The EC50 doses were compared to determine fold change. Data are represented as mean +/- SEM.

3.3.4 Concluding remarks

The results from this study indicate that carboplatin is equally if not more gonadotoxic than cisplatin; therefore, inclusion of carboplatin in treatment regimens may not offer any additional protection from chemotherapy-induced damage to the gonads. Following publication, these results have been confirmed in a human fetal testis culture model. Finally, work here shows that cultured mouse prepubertal gonads can be useful in reproductive toxicity studies to update and provide evidence for the gonadotoxicity classification of drugs used to treat childhood cancer.

Chapter 4. Vincristine-induced Damage to the Prepubertal Mouse Testis

4.1 Introduction

Vincristine belongs to the Vinca alkaloid class of chemotherapy drugs and acts as a microtubule-targeting agent: it was approved by the FDA in 1963 for cancer treatment. The vinca alkaloids are derived from the *Catharanthus roseus* plant of the Apopcynacae family, commonly known as the Madagascar periwinkle plant and has been used as a medicinal herb in Chinese medicine (reviewed in Martino *et al.*, 2018). Vincristine is currently considered safe for use in paediatric patients in regards to later fertility from clinical studies finding this drug a low gonadotoxic agent (reviewed in Wyns *et al.*, 2010; Allen *et al.*, 2018, see Table 1.1). As is so often the case, these conclusions are derived from clinical studies where patients received a cocktail of chemotherapy drugs to treat the cancer, which can make it difficult to determine the relative contributions of each drug to the gonadotoxicity prolife of a treatment regimen. With regards to vincristine gonadotoxicity, studies in prepubertal animal model systems are limited, as described in Chapter 1.5.4, with studies so far conducted in the GC-6spg SSC cell line which found a dose-dependent decrease in cell viability (Beaud *et al.*, 2017). Vincristine was chosen for investigation in this PhD study because this drug is not thought to induce oxidative stress, and was therefore considered to be a good 'negative' control for experiments to assess the role of oxidative stress-induced damage following cisplatin and doxorubicin treatment (reviewed in Conklin, 2004).

4.1.1 Mechanism of action

Vincristine is a first generation drug of the vinca alkaloid class and, alongside vinblastine, was one of the earliest chemotherapy agents discovered to target microtubules. These drugs were therefore investigated for their anti-mitotic activity and were found to disrupt cellular proliferation of cancerous cells (reviewed in Risinger *et al.*, 2009; Dumontet and Jordan, 2010; Martino *et al.*, 2018). Microtubules play important roles within the cell as they form components of the cytoskeleton and

during mitosis are essential for chromosome segregation and cell division. Microtubules are composed of α and β tubulin subunits that form heterodimers that polymerise head to tail, forming protofilaments that align in parallel to each other creating a hollow tube of 24nm diameter (reviewed in Nogales, 2001) (Fig 4.1A). Microtubules are polar structures with a plus (+) end with the β-tubulin exposed and a minus (-) end with the α-tubulin exposed: new heterodimers are added to the (+) end with irreversible hydrolysis of GTP in the β-tubulin subunit to GDP releasing energy; α-tubulin contains GTP but this is non-hydrolyzable (reviewed in Risinger *et al.*, 2009). The microtubules are dynamic structures since they can quickly reorganise themselves through addition (polymerisation) or removal (depolymerisation) of tubulin heterodimers at the (+) end, with the rate dictating whether the microtubule grows or shrinks, known as dynamic instability (Mitchison and Kirschner, 1984). The microtubules can also stabilise in length with loss of heterodimer tubulin units at the (-) end and gain of tubulin at the (+) end, known as treadmilling (Margolis and Wilson, 1978). Drugs within the vinca alkaloid class bind to microtubules at the vinca domain located in the (+) end of the microtubule, at the region of the GTP binding site of β-tubulin. Binding interferes with hydrolysis of GTP and can result in conformational changes to the tubulin, potentially disrupting overall structure and ability of microtubule-associated proteins (MAPs) to bind to the microtubule (reviewed in Downing, 2000; Dumontet and Jordan, 2010; Stanton *et al.*, 2011). The action of vinca alkaloids upon microtubules is dose-dependent, with low, sub-micromolar concentrations binding directly to the tubulin at the (+) end and inhibiting dynamic instability; these drugs are commonly considered as 'end poisons' (Toso *et al.*, 1993; reviewed in Martino *et al.*, 2018). Higher concentrations result in vinca alkaloids binding to free tubulin heterodimers, forming paracrystalline aggregates and causing active depolymerisation of the microtubule (Wilson *et al.*, 1982; reviewed in Martino *et al.*, 2018) (Fig 4.1). Vinca alkaloids can disrupt mitosis when the microtubules reorganise to form the mitotic spindles under control of the centrosomes, known as the mitotic spindle organising centres (MTOCs), found at the polar ends of the nucleus. These MTOCs contain the γ-tubulin ring complex which acts as a 'seed' to

start off nucleation/polymerisation of the α/β-tubulin subunits to form the microtubules (reviewed in Teixidó-Travesa *et al.*, 2012). During metaphase, the mitotic spindles attach to the kinetochores of the chromosomes that align along the metaphase plate and are separated by depolymerisation of the mitotic spindles, pulling the chromosomes to the opposite poles during anaphase (reviewed in Stanton *et al.*, 2011). Vinca alkaloid treatment, however, can result in failure of the mitotic spindles to form, which arrests mitosis at pro-metaphase during the spindle assembly/metaphase-anaphase checkpoint; this can then lead to activation of multiple signal transduction pathways, which can in turn lead to apoptosis (Okouneva *et al.*, 2003; reviewed in Dumontet and Jordan, 2010; Martino *et al.*, 2018) (overview of microtubules and vincristine action Fig 4.1). Signal transduction pathways activated include the c-jun N-terminal kinase/stress-activated pathway, which is activated by Ras kinases and apoptosis signal regulating kinases (Wang *et al.*, 1998; Shtil *et al.*, 1999). Studies have also shown enhanced activity of caspase 3 (Toh *et al.*, 1998) and Raf-1 kinases that leads to phosphorylation of Bcl-2 and/or Bcl-xL, which inhibits its function in preventing apoptosis (Blagosklonny *et al.*, 1997; Poruchynsky *et al.*, 1998). Further research, however, is required to fully understand the underlying molecular mechanism that results in cell death following failure to pass through the spindle assembly checkpoint during mitosis (reviewed in Rieder and Maiato, 2004).

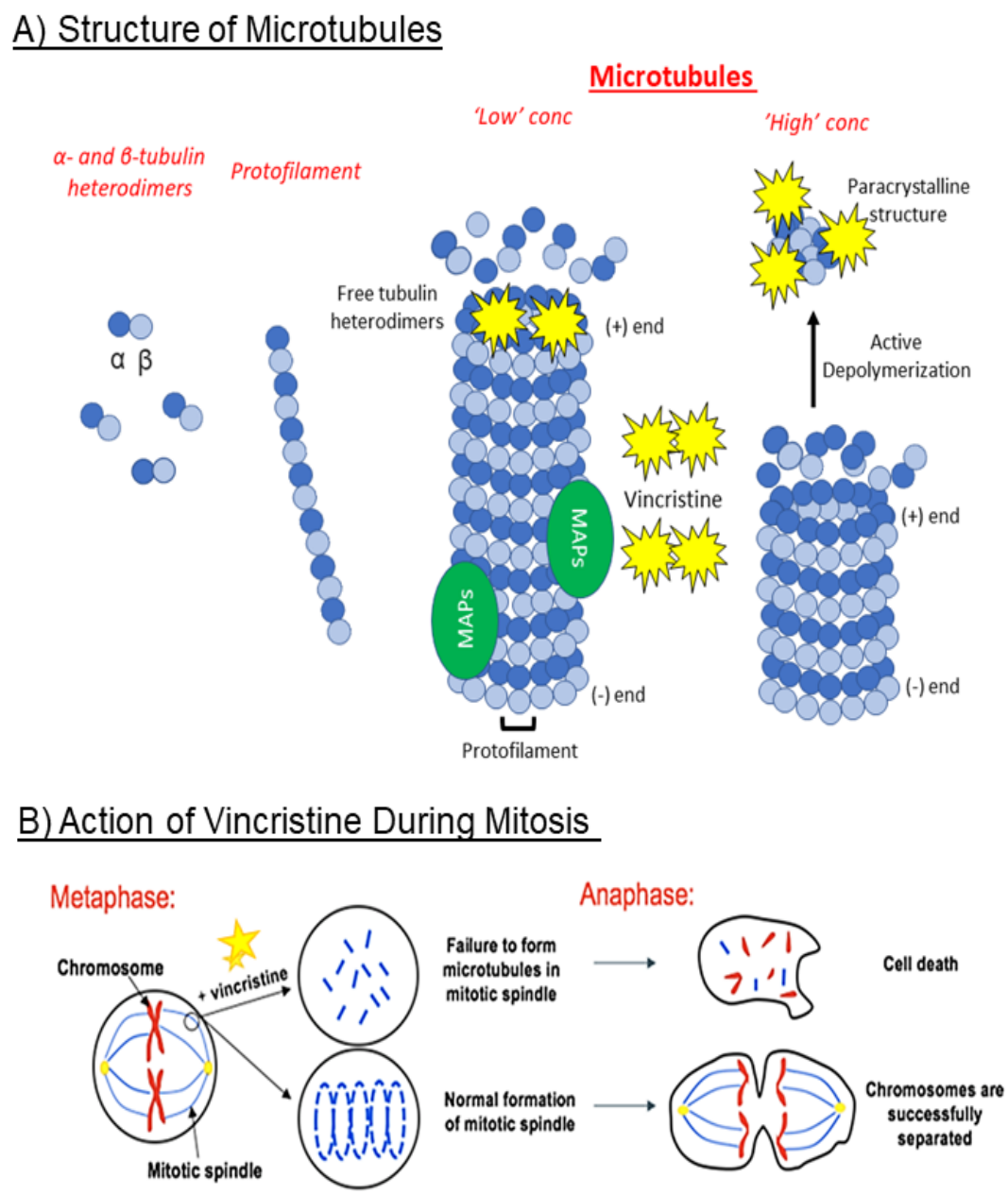


Figure 4.1: Structure of Microtubules and Action of Vincristine. A) Microtubules are composed of α and β tubulin subunits which form heterodimers. The heterodimers polymerise to form protofilaments that align themselves in parallel to form a hollow tube which constitutes the microtubule. Vincristine action is dose-dependent with vincristine binding to the vinca alkaloid site on the plus (+) end of the tubule at low doses and at high doses forming paracrystaline structures and stimulating active depolymerisation. B) Vincristine, during mitosis, binds to the mitotic spindles and prevents proper formation that disrupts chromosome segregation. Mitosis is halted at metaphase with cells ultimately undergoing cell death processes. (A) Image prepared by C.Allen and modified from Martino *et al.* (2018) and (B) designed by A. Parveen (honours student). MAPs: microtubule associated proteins. Low and High conc in reference to vincristine.

Classically, the vinca alkaloids were considered to be M-phase-specific chemotherapy agents that disrupt mitosis by targeting the microtubules, however, researchers have debated whether the vinca alkaloids can also have activity outwith the M-phase of the cell cycle (reviewed in Komlodi-Pasztor *et al.*, 2011). Studies into microtubule dynamics following vinca alkaloid treatment have been performed using purified tubulin heterodimers in solution, however, such free tubulin heterodimers are not normally found *in vivo* (reviewed in Jordan and Kamath, 2007). Research into their therapeutic potential has mainly been conducted in cell culture and xenograft experiments where cells undergo rapid cell division, however, the vinca alkaloids can be used to treat slow growing tumours. In addition, research focusing upon other known anti-mitotic agents, such as aurora kinase inhibitors, as a cancer treatment has not been as successful as studies with vinca alkaloids (reviewed in Komlodi-Pasztor *et al.*, 2011). This would therefore suggest that the vinca alkaloids can target microtubules outwith the M-phase of the cell cycle, which would disrupt normal cellular functions, potentially contributing to the cytotoxic nature of these drugs. Cellular processes which involve the cytoskeleton could be affected by vinca alkaloid treatment and include disruption to cellular transport, signalling, migration, metabolism, secretion, overall structure and wound healing (reviewed in Komlodi-Pasztor *et al.*, 2011; Bates and Eastman, 2017). Further research into these possible additional mechanisms is required (Howard *et al.*, 1980; Jordan *et al.*, 1991)

4.1.2 Clinical use and pharmodynamics

Vincristine is commonly used to treat paediatric/childhood cancers, specifically haematological malignancies such as acute lymphocytic leukaemia, Hodgkins and non-Hodgkins lymphoma as well as Ewing sarcoma, Wilms tumor, neuroblastoma and rhabdomyosarcoma (Rowinsky, 2003). This drug is included in multiple different combination therapies including CVP (cyclophosphamide, vincristine and prednisone) and CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), both to treat follicular B-cell lymphoma (reviewed in Martino et al., 2018). The main dose-limiting side effect of vincristine treatment is neurotoxicity, with damage to the

peripheral nervous system due to axonal degeneration and decreased axonal transport as a result of disruption to microtubule function, which can cause severe sensory and motor neuropathies (reviewed in Mora *et al.*, 2016). Vinca alkaloids are given to patients through intravenous infusions, with the drugs rapidly diffusing into peripheral tissues and cells; sensitivity of individual tissues/cells is dependent on multiple factors including; tubulin isotypes, MAPs and GTP. Plasma half-life for vincristine is 23-85hrs, with drugs detoxified in the liver through action of cytochrome P450 (CYP)3A, with the less toxic metabolites cleared through biliary elimination and excreted into faeces (reviewed in Martino *et al.*, 2018). The clinical pharmacology and determination of plasma serum levels of vincristine has mainly been performed by studies in the 1990s and early 2000s, and so techniques analysing concentrations may not be as sensitive compared to current techniques (overview in Rowinsky, 2003). Adult and paediatric patients normally receive doses of 1.5 - 2mg/m^2, with plasma serum levels peaking at 0.5µg/ml, and with slower clearance than other vinca alkaloids due to higher tissue retention due to lipophilicity (Nelson *et al.*, 1980; Crom *et al.*, 1994; Rowinsky, 2003). In paediatric patients, clearance rates may be higher, with studies finding steady state concentrations of up to 0.005µg/ml (Crom *et al.*, 1994; Kellie *et al.*, 2004).

4.1.3 Aims and hypothesis

This chapter aims to examine the impact of vincristine treatment on the male prepubertal reproductive system through use of our prepubertal testis culture model. My initial hypothesis was that vincristine treatment would have little impact on the prepubertal testis due to the current clinical classification of this drug as a 'low risk' gonadotoxic agent (Wyns *et al.*, 2010). To the best of my knowledge, there have been no such studies to date that have been conducted with prepubertal tissue. Results in this chapter provide the dose-response for vincristine treatment required for further work in Chapter 5, which utilizes vincristine as a 'negative control' drug when investigating the role of oxidative stress in chemotherapy-induced damage to the prepubertal testis.

4.2 Methods

4.2.1 Experiment 1: Establishing a dose-response for vincristine

The dose-response for vincristine was determined by exposing the testis fragments on day two of culture to a wide range of concentrations of vincristine including 0.001, 0.01, 0.1, 1 or 10µg/ml for 24hrs. In this initial work, clinical preparations of the vincristine drug were used to determine the dose-response, with the manufacturer providing information on the diluents (known as the excipients) used. The clinical preparation contained the excipients mannitol, sulphuric acid and sodium hydroxide for pH adjustment, and water for injections. Mannitol is a hexahydric alcohol present in sweeteners and is used clinically as a tonicity agent in liquid drug preparations (reviewed in Raymond C *et al.*, 2012; Rayaprolu *et al.*, 2018). Concentrations for the excipients, however, were not available from the data sheets; neither could that information be obtained upon request. The stock solution of vincristine was 1mg/ml with working concentrations added to the culture plate as described in Chapter 2 (see 2.2). Control tissues were exposed to equivalent volumes of water instead of the vincristine drug. Experiments were performed with six litters from five culture runs, therefore total 'n'=6. One tissue fragment per each experimental group was analysed per culture (total 'n'=6). The cultures were performed by myself, with the microtoming, staining and analysis undertaken by an honours student ('n'=4), Aisha Parveen, who I supervised on a day-to-day basis. Additional microtoming, staining and analysis was performed by myself for the final 'n'=2.

4.2.2 Experiment 2: Role of excipients in vincristine-induced damage from clinical preparation

4.2.2.1 Preparation and use of mannitol in prepubertal testis culture

The potential impact of the excipients in the clinical preparation on the prepubertal testis was assessed to determine whether results obtained from the dose-response were due to the action of the vincristine drug itself, or whether they could be due to the excipients. The prepubertal testis fragments were cultured and exposed to selected concentrations of the excipient mannitol. As outlined above, the

concentration of mannitol in the clinical drug preparation is unknown; however, a review by Rayaprolu *et al.* (2018) states that this excipient is "commonly used at 5%". A solution of 5% (w/v) was therefore used as our highest stock concentration, which would result in the tissues being exposed to a maximum working concentration of 0.05% (1:100 dilution from 5% stock based upon vincristine dilution). Vincristine was included for comparison at the highest working concentration used within our dose-response study of 10µg/ml, which was prepared from the 1mg/ml stock solution (1:100 dilution of 1mg/ml stock to give 10µg/ml working concentration). Stock concentrations of mannitol were prepared (w/v) with mannitol (Sigma, UK, M4125) dissolved in distilled water, solutions were sterilised with bottle top vacuum filters before use. The prepubertal testis fragments were exposed to either 0.0025, 0.0125, 0.025 or 0.05% mannitol or 10µg/ml vincristine for 24hrs on day two of culture. Control tissues were exposed to equivalent concentrations of water. This culture was performed by myself with the tissues microtomed, stained and analysed by honours project student Aisha Parveen who was under my supervision. This culture was performed once ('n'=1) before moving on to different approach as outlined below.

4.2.2.2 Comparison of the effects of powdered and clinical preparations of vincristine on prepubertal testis

The concentrations of the excipients in the clinical preparation of the vincristine are unknown, with the concentrations of mannitol used in the above culture (4.2.2.1) hypothesised from the literature. Further cultures were therefore set up to compare powdered vincristine made up in water (see 2.2.1) to the clinical preparation of vincristine. The tissues were exposed to 0.01, 0.1 or 1µg/ml of either powdered vincristine or clinical vincristine on day two of culture for 24hrs. The control tissues for these cultures were exposed to equivalent volumes of water with the same control for both the powder and clinical preparation. This culture was performed twice ('n'=2), with inclusion of one run alongside a dose-response culture.

4.2.3 Experiment 3: Determining whether the damage in the four day culture is observed after two weeks of culture of the prepubertal testis tissues

The long-term effects of acute exposure to vincristine, on day two of culture for 24hrs, was assessed to determine whether there is any capacity for the tissue to recover after long-term culture (two weeks) of treated tissues. For long-term cultures, testes were collected from pnd5, CD1 mice and dissected into smaller fragments than for previous cultures (2.1.2), resulting in fragments of roughly 0.5mm^3 in size. Culturing of larger fragments (1mm^3) for longer periods of time results in necrosis where the tissue grows in size and cannot be supported by this culture method (personal communication, Dr Federica Lopes). Due to the longer culture duration increasing the chance of infection, the culture medium of αMEM and 10% KSR was supplemented with 1% antibiotic-antimycotic (penicillin streptomycin - amphotericin B) (Thermofisher, 15240096). Cultures were setup as previously described in Chapter 2 with the testis tissues exposed to 0.05 or 0.1µg/ml vincristine on day two of culture for 24hrs before fresh, drug-free media change. The vincristine used for these experiments was the powder form dissolved in DPBS, with the working concentrations chosen based on the dose-response experiment (4.2.1), ensuring that the dose was not too high, to increase the potential for recovery. Control tissues were exposed to equivalent volumes of DPBS. The media was changed every two/three days throughout the culture. BrdU (1.5µg/ml) was added for the final 24hrs of culture to compare proliferation rates. Tissues were collected as stated in Chapter 2 from each experimental group on day four (short-term, as cultured in 4.2.1/2) or on day fourteen/week two (long-term) to determine the long-term impact of acute vincristine exposure. Cultures were performed by myself, with an 'n' of four from two culture runs, with a plan to increase sample size further if required. The microtoming, staining and analysis was performed by an honours student, Catherine Stewart, who was under my day-to-day supervision. Due to laboratory closure resulting from Covid-19, tissues were processed and analysed from only 'n'=2.

4.2.4 Immunofluorescence

The additional primary and secondary antibodies as well as dilutions used within this chapter are shown in Table 4.1 and 2.1. For experiments one and two, double immunofluorescence with MVH and BrdU was used to assess proliferating germ cells. For experiment 3, double immunofluorescence with MVH and PLZF enabled assessment of the germ cell and SSC populations.

Table 4.1: Primary and secondary antibodies for immunofluorescence

Primary Antibody	Primary Ab Dilution	Secondary Antibody (diluted 1:500)
Rabbit anti-PLZF (Abcam, ab189849)	1:4000	Alexafluor goat anti-rabbit IgG 488nm

4.2.5 Analysis

Manual counting was undertaken to assess the germ cells. Following exposure to vincristine, however, it was no longer possible to properly outline the seminiferous tubules by eye. All analysis was therefore undertaken determining germ cell density per section area (cell numbers/mm^2).

4.3 Results

4.3.1 Dose-response of vincristine-induced damage to prepubertal testis

In order to assess the impact of vincristine treatment on the prepubertal testis, a dose-response was first determined by exposing cultured testis fragments to a range of concentrations of a clinical preparation of the vincristine drug from 0.001 – 10µg/ml. The number of germ cells (MVH+) and proliferative capacity of these cells (BrdU+ or -) were assessed through manual counting. Overall, vincristine treatment had a considerable effect on the density of the germ cell population, with the control population significantly different compared to 0.1µg/ml (71% decrease, p=0.0098), 1µg/ml (61% decrease, p=0.0238) and 10µg/ml (74% decrease, p=0.0015) vincristine-exposed tissues (Fig 4.2, Fig 4.3i, 'n'=6). Vincristine specifically reduced the proliferating germ cell population (MVH+, BrdU+) at 0.1µg/ml (94% decrease, p=0.0018), 1µg/ml (89% decrease, p=0.0089) and 10µg/ml (74% decrease, p=0.0219)

(Fig 4.2, Fig 4.3ii, ‘n’=6). There was no effect when comparing the control density of non-proliferating germ cell population, 617+/-55 germ cells per mm^2, compared to vincristine-exposed tissues at the concentrations tested (Fig 4.2, Fig 4.3iii, p=>0.05, ‘n’=6). The testicular architecture following vincristine treatment with concentrations at or above 0.1µg/ml was disrupted compared to control testes. The PMCs in particular were hard to identify, making it difficult to visualise seminiferous tubules. For this reason, area of tissue rather than areas of seminiferous tubules were calculated (Fig 4.2/4.3/4.4). Following vincristine treatment at concentrations above 0.1µg/ml, there was evidence of cellular degeneration based on the observation of cells with acidic cytoplasm and condensed DNA (Fig 4.4A). In addition, accumulation of germ cells within the centre of seminiferous tubules was apparent, with the cell shape and size varying with expanded and shrunken morphology noted (Fig 4.4). These shrunken cells could be representative of individual germ cells or of fragmented nuclei from germ cells (Fig 4.4; white arrowhead). These central germ cells were present at the end of the culture period, which is after a two-day recovery period.

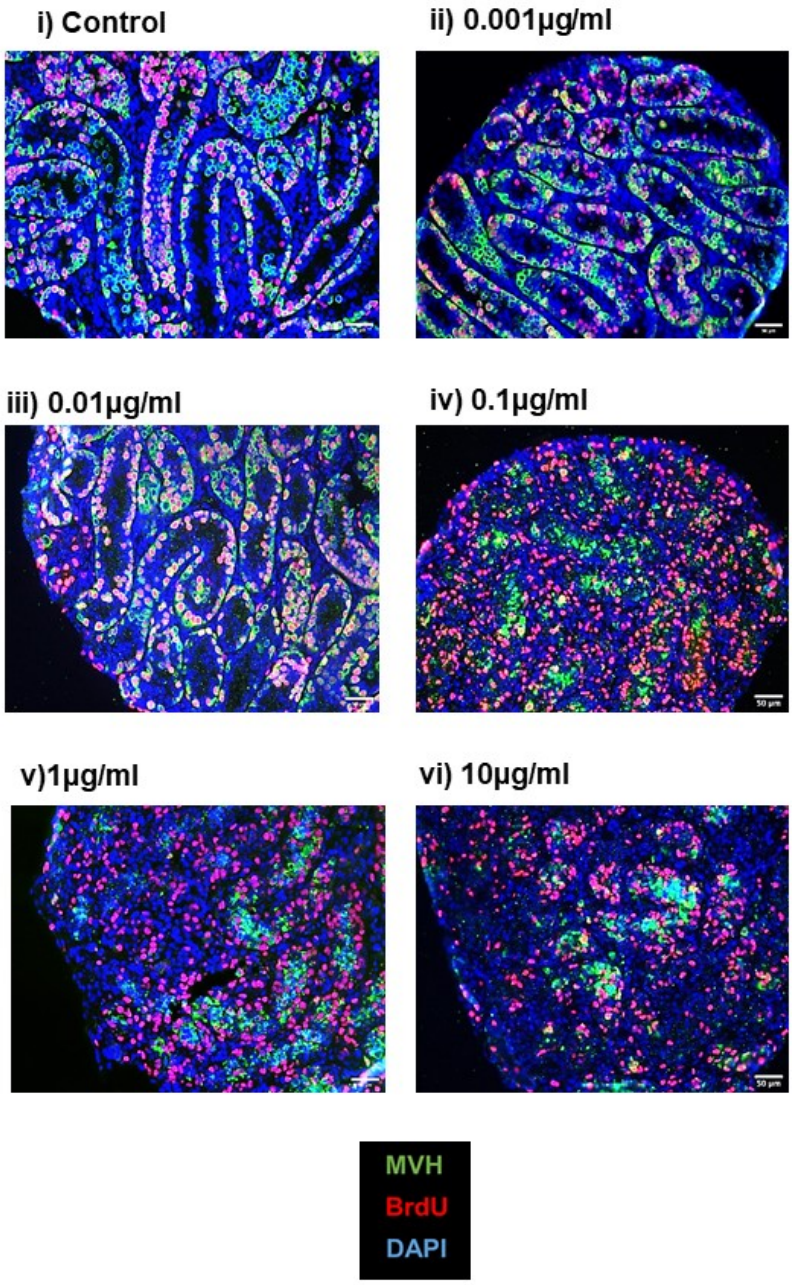


Figure 4.2: Representative images of dose-response of testis exposed to 0.001-10µg/ml vincristine. (i) control and vincristine treated tissues at concentrations from (ii) 0.001µg/ml to (vi) 10µg/ml. Images were taken at 20X for analysis and scale bar = 50µm. 'n'=6.

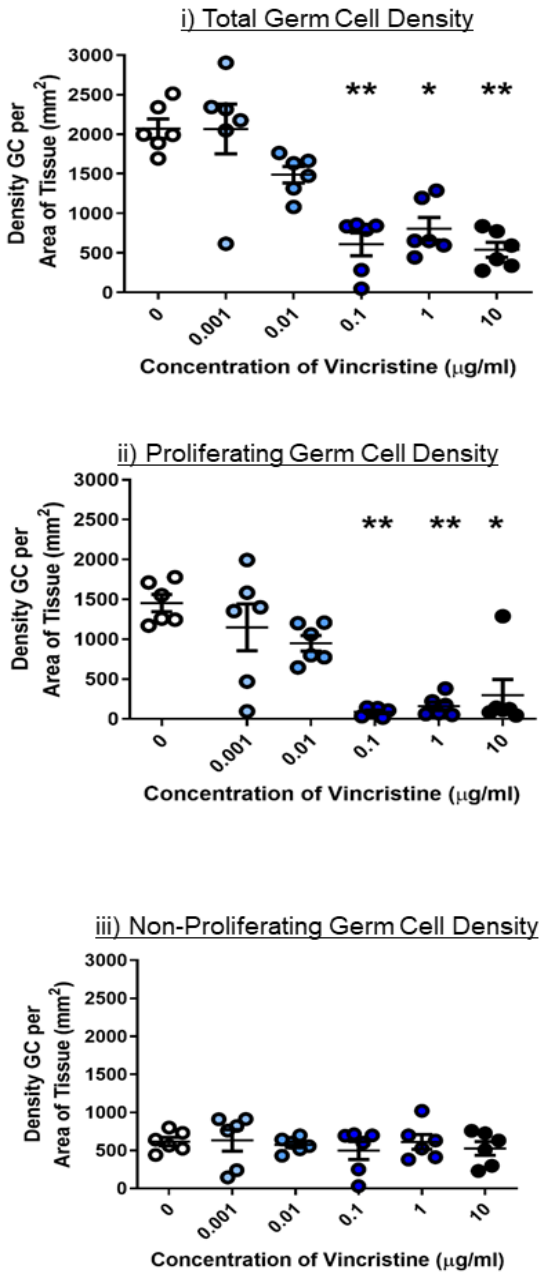


Figure 4.3: Analysis of dose-response of testis exposed to 0.001-10µg/ml vincristine. (i) Germ cells (green MVH+) were manually counted per tubule area and classified as (ii) proliferating (green MVH+, red, BrdU+) or (iii) non-proliferating (green MVH+, BrdU-). The density of the total number and proliferating germ cells decreased with increasing concentrations of vincristine, with germ cells located in the centre of the seminiferous tubules. 'n'=6. See Appendix C Figure 1 for QQ-plots analysing normal distribution. Data analysed by Kruskal-Wallis tests followed by Dunn's multiple comparison test for total and proliferating germ cells. Data analysed with one-way ANOVA followed by Dunnetts post-hoc test for non-proliferating germ cells. *p<0.05, ** p<0.01, comparing treatment groups to the control. Data are shown as mean +/- SEM.

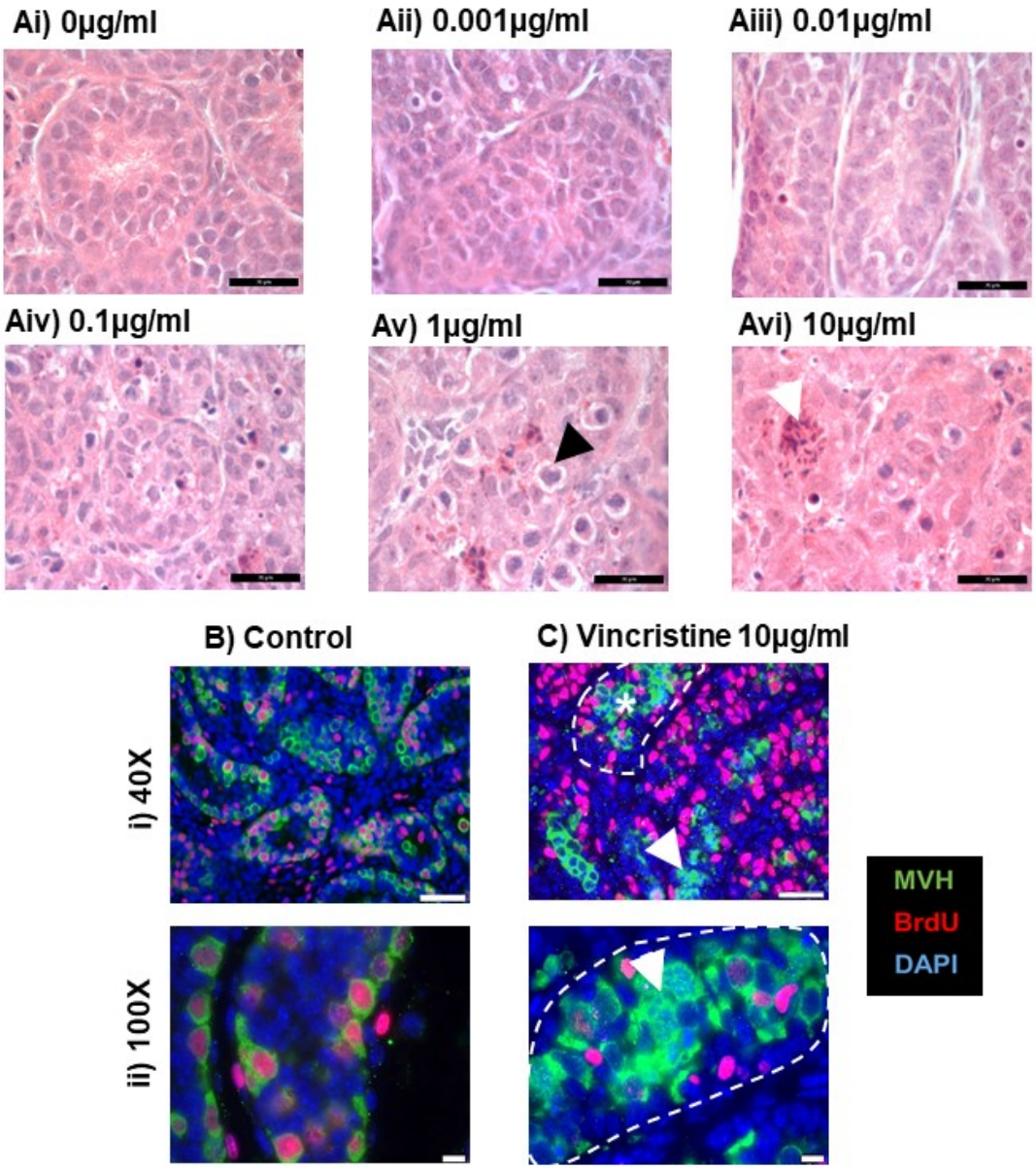


Figure 4.4: Morphology of testis following vincristine treatment. Representative images from (A) H&E stained sections and (B/C) immunofluorescent images showing central clumping of misshapen germ cells (green, MVH+) in the (C) vincristine 10µg/ml treated tissues compared to (B) controls. (Aiv, v & vi) Cell death apparent in the H&E stained images following vincristine treatment. Black arrow example of enlarged cell/nucleus, white arrow example of a clump of germ cells (cells in H&E) which could represent individual cells or nucleus fragmentation. White star example of accumulated germ cells in the centre of proposed seminiferous tubule outlined by dashed white line. A) Images taken at 100X, black scale bar representative of 20µm, B/Ci) white scale bar 50µm and B/Cii) white scale bar 10µm.

4.3.2 The excipient mannitol that is used in the clinical preparation of vincristine does not affect testis morphology.

The clinical preparation of the drug vincristine contains the excipient mannitol, although at an unknown concentration (the company would not provide this information), and therefore the concentration that the tissues in experiment 4.3.1 were exposed to are unknown. In order to investigate whether mannitol could induce the effects seen in experiment 4.3.1 above, a pilot dose-response experiment was performed examining effects of concentrations of mannitol ranging from 0.0025-0.05%. These concentrations were chosen with the assumption that mannitol concentrations were around 5% in the 1mg/ml stock solution, as indicated by a literature search (reviewed in Rayaprolu *et al.*, 2018). Given that 'n'=1 for this experiment, it is of course not possible to carry out any statistical analysis: none-the-less there is no indication of any effect of mannitol exposure at the concentrations tested, when looking at the density, proliferative capacity or morphology of the seminiferous tubules or germ cells (Fig 4.5/4.6). This is in contrast to the marked effects observed when testis tissues were treated with 10µg/ml vincristine (Section 4.3.1 above and Fig 4.5ii). The results suggest that mannitol was not gonadotoxic at these concentrations, with the density of the germ cells following mannitol treatment 1799 +/- 108 germ cells per tubule area across the treatment groups compared to the 2311 germ cells per tubule area noted in the control tissue (Fig 4.6). Given that it is not possible to be certain whether the concentrations tested here represent concentrations in the clinical preparation of the drug, further repeats of this culture were not undertaken, and an alternative strategy was employed (4.3.3).

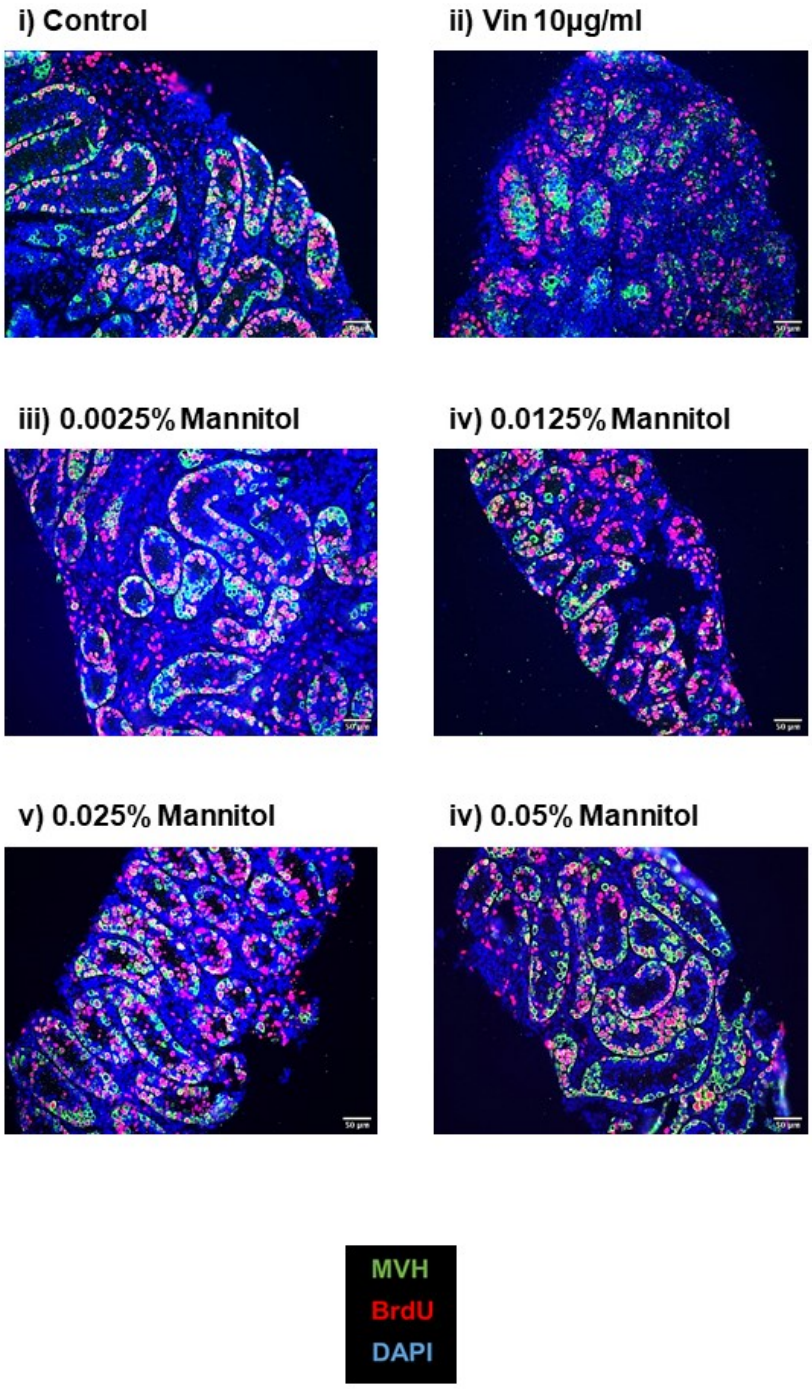


Figure 4.5: Representative Images of effects of excipient mannitol on the prepubertal testis. (i) control, (ii) 10µg/ml vincristine treated tissues with central germ cell clumping and (iii-iv) mannitol exposed tissues that were treated with 0.0025% to 0.05% mannitol. 'n'=1, images taken at 20X and scale bar = 50µm.

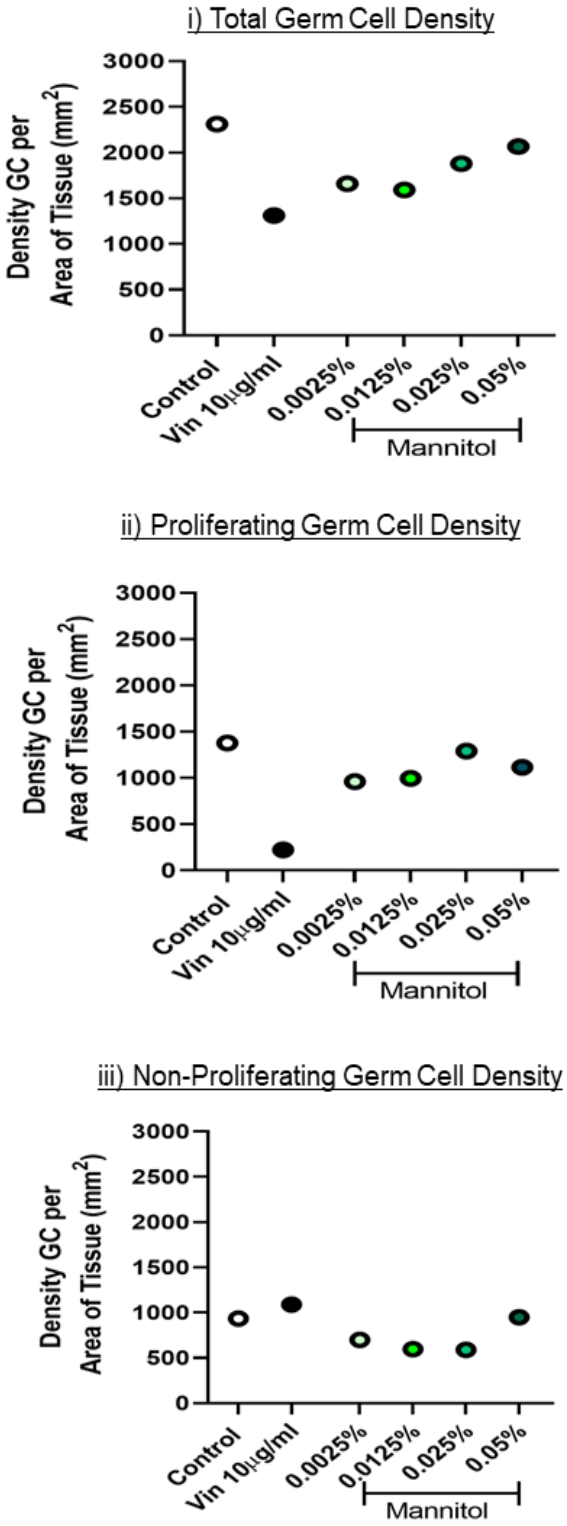


Figure 4.6: Analysis of effects of excipient mannitol on the prepubertal testis. (i) Total germ cell density (green, MVH+), (ii) proliferating (green MVH+, red BrdU+) and (iii) non-proliferating germ cell density was not found to be altered following mannitol treatment in this culture with tissues comparable to control in location of germ cells (MVH+). 'n'=1.

4.3.3 Clinical and powder preparations of the vincristine drug have the same effect upon the prepubertal testis

A different approach to determine whether the effects of vincristine noted with the clinical preparation above (4.3.1) were due to the action of the drug or the excipients, mainly mannitol, was undertaken by comparing the effects of clinical to powdered vincristine. Prepubertal testis fragments were exposed to 0.01, 0.1 or 1µg/ml of either clinical vincristine or powdered vincristine (made up in distilled water and so with no additional constituents present). These concentrations were chosen as they had previously been found to decrease proliferating germ cell density and result in central clumping of germ cells. Germ cells were clumped within the centre of the seminiferous tubules after exposure to either the powder form or clinical preparation of the drug (Fig 4.7/4.8). Difference at 0.01µg/ml where noted, this could be due a contribution of mannitol at lower vincristine doses or due to discrepancy/user error when preparing the vincristine drug. Overall, there is no suggestion that there were differences between the clinical and powdered form of the drugs when comparing germ cell densities. Due to the low 'n' number for this experiment, statistical analysis could not be undertaken, however, the results from these two cultures and the above culture (4.3.2) provides evidence that the damage following vincristine treatment is due to the action of the drug itself rather than to any excipient present.

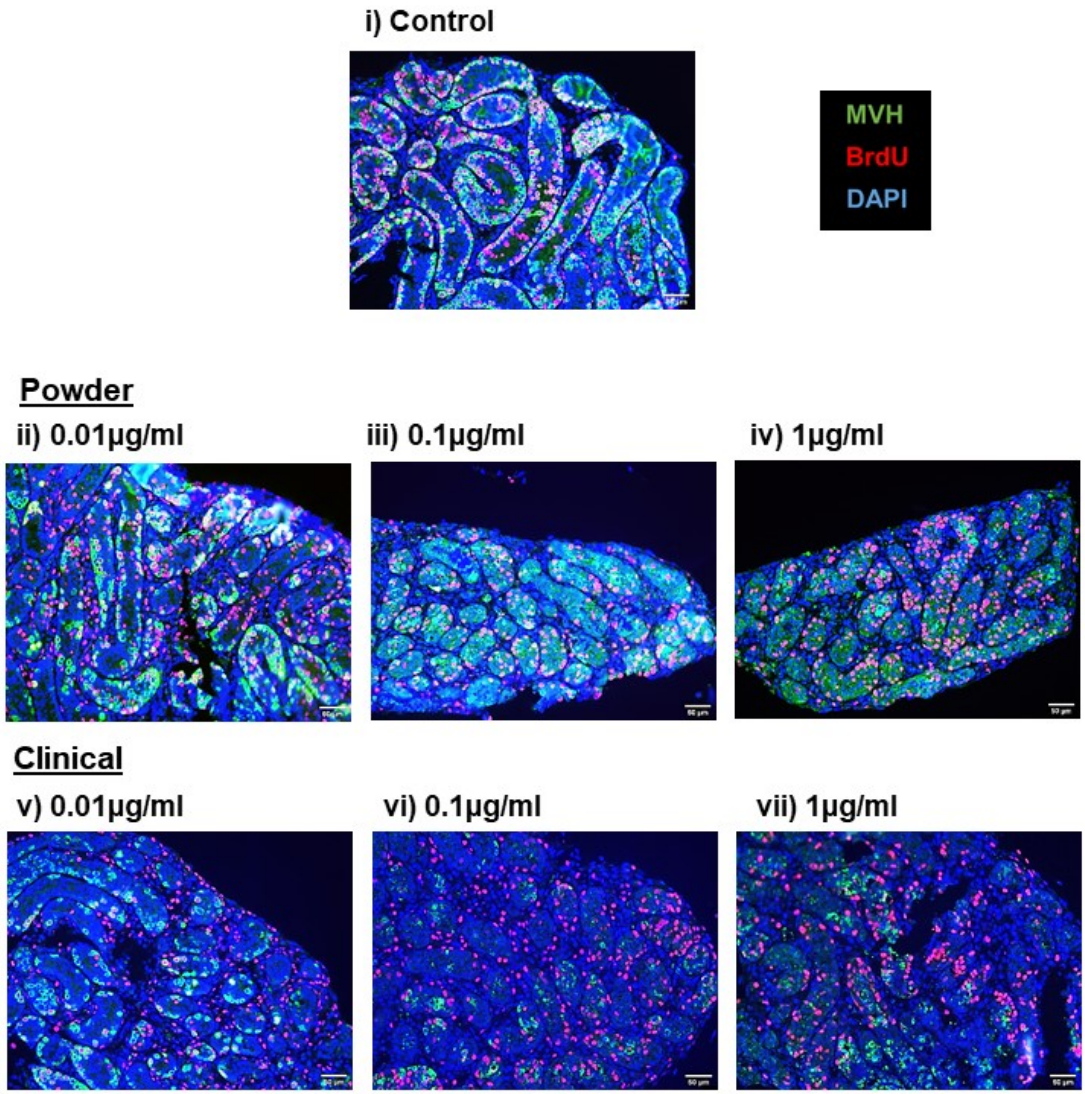


Figure 4.7: Powder vincristine has same effect as clinical preparation of vincristine – representative images. (i) control and vincristine treated tissues with (ii-iv) powder and (v-vii) clinical forms of the drug at (ii, v) 0.01, (iii, vi) 0.1 and (iv, vii) 1µg/ml vincristine. Clumping of germ cells in centre of seminiferous tubules are noted at 0.1 and 1µg/ml. 'n'=2, images taken at 20X and scale bar = 50µm.

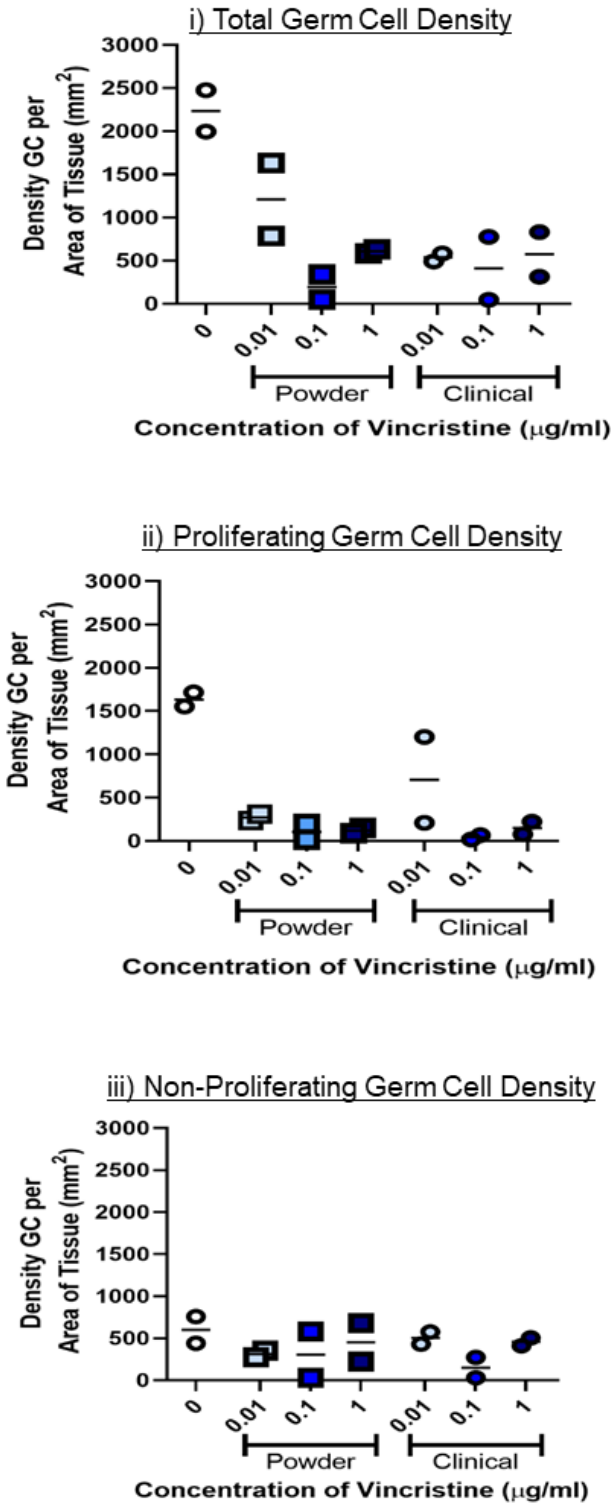


Figure 4.8: Powder vincristine has same effect as clinical preparation of vincristine – analysis. (i) Total germ cell density (green, MVH+), (ii) proliferating (green MVH+, red BrdU+) and (iii) non-proliferating (green MVH+, BrdU-) germ cell density comparable between powder and clinical forms of drug. 'n'=2.

4.3.4 No evidence of recovery of germ cell population after two weeks of culture following acute 24hr exposure to vincristine.

The observation of a population of germ cells within the centre of the seminiferous tubules two-days after a 24hr period of exposure to vincristine, raised the question of whether these germ cells could survive, and if so whether they would be capable of repopulating the germ cells within the testis if allowed time to recover. Long-term testis cultures for two weeks were therefore undertaken and compared to the short-term culture of four days, which was the conditions in which the initial observation had been noted above (4.3.1). Tissues were treated with 0.05 or 0.1µg/ml vincristine and collected either two-days later (short-term culture) or twelve days later (long-term culture). The number of germ cells (MVH+) and number of SSCs (PLZF+) were manually counted per tissue section area. Due to complications arising from Covid-19, an 'n' of two was analysed for this experiment. From the results analysed ('n'=2), the germ cells formed clumps within the centre of the seminiferous tubules following vincristine treatment in the short-term culture but were absent in the long-term cultures (Fig 4.9A). The SSC population was analysed, as maintenance of SSCs is essential to repopulate the total germ cell population. Because the PLZF antibody gave a positive signal outside the seminiferous tubules in the interstitium, only PLZF+ cells that were also MVH+ were counted. The data collected indicates that there are fewer SSCs in the vincristine treated tissues in the short- and long-term cultures, and those remaining were physiologically located at the basement membrane of the seminiferous tubules, i.e. within the SSC niche as expected. There appears to be fewer germ cells and SSCs in the control tissues of the long-term culture in comparison to the short-term culture, this may be due to an effect of culture or due to the low 'n' number obtained for this experiment.

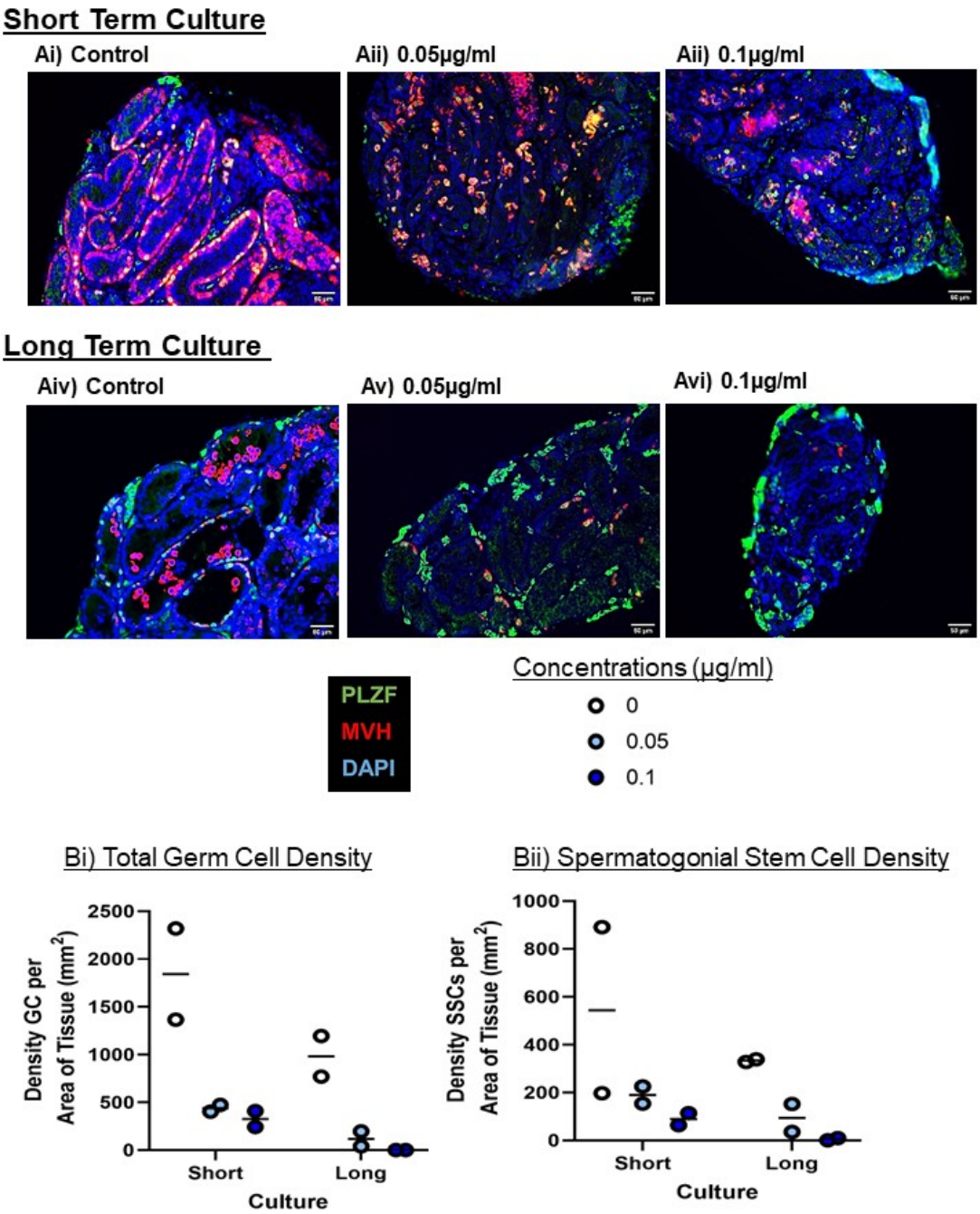


Figure 4.9: Long-term impact of vincristine treatment on the prepubertal testis. (A) Representative images of (Ai-ii) short-term and (Aiv-vi) long-term culture of testis fragments following 24hrs of vincristine treatment at (Aii, v) 0.05 and (Aiii, vi) 0.1µg/ml. Germ cell clumping within the short-term vincristine treated tissue are no longer present in the long-term cultures. The density of (Bi) germ cells (MVH+) and (Bii) SSCs (MVH+, PLZF+) per tissue section area where reduced following vincrisitne treatment in both short and long-term cultures. 'n'=2, images taken at 20X and scale bar = 50µm.

4.4 Discussion

4.4.1 Overview of results

In this study, prepubertal testicular fragments were exposed to a range of concentrations of clinical preparation of vincristine for 24hrs to determine a dose-response for this tissue. Vincristine is currently considered a 'low risk' gonadotoxic agent in humans (reviewed in Wyns *et al.*, 2010, see table 1.1), and therefore it was hypothesised that exposure would have no observable impact on the prepubertal mouse testes within this culture system. Based upon the results, however, this hypothesis can be rejected since vincristine had a significant impact on the prepubertal mouse testis when damage was assessed 48hrs after treatment. The density of the proliferating germ cells were decreased at the high concentrations tested (0.1, 1 and 10µg/ml; $p<0.0001$), with accumulation of germ cells in the centre of the seminiferous tubules. Due to the use of a clinical preparation with unknown concentrations of excipients in the first experiment, it was not possible to determine whether the results obtained were due to the action of vincristine or due to any excipients present, including that of mannitol. Cultures were therefore set up to assess differing concentrations of mannitol ('n'=1) or to compare powder (without excipients) and clinical preparations at the highest concentrations ('n'=2). Findings from these cultures suggest that the effects of clinical preparation of vincristine were a result of vincristine action. Due to the unusual clumping of germ cells resulting from vincristine exposure, set alongside the clinical findings of a low impact on fertility of vincristine in paediatric treatment regimens, longer-term cultures were undertaken to determine the capacity for recovery following 24hrs of vincristine exposure on day two of culture. Assessment of the germ cell and SSC populations twelve days after exposure found that accumulated germ cells were no longer present compared to the short-term cultures, indicating that the germ cells had undergone cell death (based on 'n'=2). Additional analysis with cell death markers such as CC3 or PARP are required to confirm this hypothesis. Fewer SSCs were also noted following vincristine treatment in both the short- and long-term cultures; however, the survival of germ cells aligned along the basement membrane suggests there may be potential for

recovery of the germ cell population, as long as some SSCs remain in the stem cell niche following vincristine exposure.

4.4.2 Dose-dependent effects on prepubertal germ cell population

The effects of vincristine upon the prepubertal testis tissue have, to this author's knowledge, yet to be examined, possibly due to the fact that it is currently considered to have 'low' gonadotoxicity (reviewed Wyns *et al.*, 2010). Despite that clinical view, in the mouse experiments here, a dose-dependent decrease in the proliferating germ cell population was found following exposure to higher concentrations of vincristine (0.1, 1 and 10µg/ml), with proliferation assessed through BrdU uptake during the final 24hrs of culture. There was no difference in the density of the non-proliferating germ cell population within the concentration range tested in these experiments. This suggests that vincristine either directly targets the proliferating germ cell population or prevents further proliferation of these cells. Vincristine could potentially target these proliferating germ cells by binding to the mitotic spindles during mitosis, which would prevent further proliferation/cell division and ultimately lead to cell death (reviewed in Jordan and Wilson, 2004). The mechanism of cytotoxicity of the vinca alkaloids have, however, been debated, given that tissues with a low proliferation rate can also be damaged following treatment; for example, vincristine treatment is associated with neurotoxicity due to action on the cytoskeleton (reviewed in Mora *et al.*, 2016; Bates and Eastman, 2017). Histological studies into the effects of vincristine treatment in adults are lacking, most likely due to the 'low' gonadotoxic classification; with the majority of studies conducted in the 1970 to 1990s in rodents, where reductions in the number of spermatogonia, primary spermatocytes, spermatids and sperm were noted (Joshi and Ambaye, 1968 as cited in D'Cruz *et al.,* 2010; Bustos-Obregon and Lopez, 1973 as cited in D'Cruz et al., 2010; Lu and Meistrich, 1979a; Hansen and Sørensen, 1991; Murugavel and Akbarsha, 1991 as cited in D'Cruz et al., 2010; Dobrzyńska *et al.*, 2005; Nikpour *et al.*, 2018). The appearance of giant germ cells has also been noted in studies in adult male rats following vincristine exposure (Stanely and Akbarsha, 1992). Rapid recovery of

spermatogenesis within three-four months of end of treatment has been described following treatment regimens which include vincristine in adults, indicating that vincristine does not damage the SSCs but targets differentiating spermatogonia or later stages of spermatogenesis to temporality arrest spermatogenesis (Meistrich *et al.*, 1997; Okada and Fujisawa, 2019).

The findings here of clumps of germ cells within the centre of seminiferous tubules days after exposure to vincristine is unusual: normally, when germ cells move into the centre of the seminiferous tubule they are undergoing cell death and are rapidly cleared from the tissue (Smart *et al.*, 2018). Apoptotic cells are generally cleared from the tissue within hrs by phagocytosis, *in vivo* taking place within 2-4hrs (reviewed in Kannan and Jain, 2000). This rapid loss of germ cells has been noted in Smart *et al.* (2018) following cisplatin or cyclophosphamide treatment eight hrs after increased CC3 expression. The timing of cell death has been debated following treatment with other mitotic inhibitors, such as chemotherapy drugs from the taxol class: these drugs are known to have similar effects as the vinca alkaloids at lower concentrations but, conversely, enhance polymerisation at the higher concentrations (reviewed in Gascoigne and Taylor, 2009). Such studies have indicated that once cells fail to pass through the spindle assembly checkpoint following taxol-treatment, cells can undergo cell death, unequal cell division or can exit the cell cycle and either be arrested in interphase, undergo cell death in interphase or enter additional cell cycles without dividing (reviewed in Gascoigne and Taylor, 2009) (Fig 4.10). An effect such as this could potentially account for the observations noted here, with the clumps of germ cells representing cells that have been arrested at mitosis and are either undergoing unequal division or exiting the cell cycle without division. This could then result in the germ cells losing contact with the SSC niche, causing the cells to migrate into the centre of the seminiferous tubule. On the other hand, loss of contact with surrounding cells due to alterations in microtubule dynamics could result in changes to the cell cycle resulting in the accumulation of germ cells. These cells would be in mitotic arrest and may potentially undergo cell death during interphase or

subsequent cell cycles, therefore long-term culture were undertaken to be discussed later (4.4.4). Time-course cultures could also be used to study this further, assessing for cell death markers or cell cycle specific markers, which would indicate whether these cells are undergoing cell death or are halted at a specific stage of the cell cycle following treatment. Within this study, BrdU uptake was analysed in the germ cell population and found to be decreased following treatment, suggesting that the germ cells here are not undergoing DNA synthesis (S-phase); further markers that could be used to determine cell cycle progression include the phospho-histone H3 marker for cells in M-phase to determine the fate of these cells. The accumulation of germ cells in the centre of seminiferous tubules is most likely due to loss of communication and/or contact with the basement membrane or the Sertoli cell population that are in physical contact with the germ cells, which have important roles in defining the SSC niche and supporting the structure and nutritional status of the germ cells (Wu *et al.*, 2020). In addition, loss of contact or communication with the PMCs that surround the seminiferous tubule may also contribute to the observations noted in this study. The PMCs play an important role in the development and function of the testis as shown in rodents studies (Welsh *et al.*, 2009; Nurmio *et al.*, 2012; Chen *et al.*, 2014). In particular, the PMCs produce extracellular matrix proteins that form the basement membrane and also produce secretory factors which enable communication with other testicular cells through paracrine signalling and is proposed to be involved in maintenance of SSC niche (reviewed in Mayerhofer, 2013). Changes to the germ cells following mitotic arrest could disrupt the connection and/or communication with the Sertoli cells and/or PMCs of the testis. Alternatively, activation of cell death pathways in the germ cells could result in loss of contact with the surrounding Sertoli cells/basement membrane, leading to their accumulation in the centre of the seminiferous tubules. Indeed, cellular degeneration is noted in H&E images of vincristine-treated tissues of centralised cells, which could be indicative of cell death. However, the presence of centrally located germ cells two-days after chemotherapy exposure suggests that these cells were not initially undergoing active cell death since this a rapid process, with cells cleared within 2-4hrs *in vivo* (reviewed

in Kannan and Jain, 2000). Within the seminiferous tubules where germ cells clump following vincristine treatment, the somatic Sertoli cells are located and have not been studied during this PhD due to time constraints. Sertoli cells contain a vast amount of microtubules forming an active cytoskeleton that is very dynamic to support movement of organelles, secretion of fluids and the shape of the cells which change to support the germ cells (reviewed in O'Donnell and O'Bryan, 2014). These cells could therefore be vulnerable to vincristine-induced damage. The Sertoli cell microtubules run parallel to each other in the long axis of the cells with the minus end pointing towards the basement membrane, this is a similar arrangement to the microtubules of axons which are known to be sensitive to vincristine-treatment (Neely and Boekelheide, 1988). Movement of germ cells into the seminiferous tubules, referred to as germ cell exfoliation, has been found in rodent model studies following microtubule disruption of the Sertoli cells (Handel, 1979; Correa *et al.*, 2002; Smith *et al.*, 2012). Following treatment, it was difficult to identify the PMC layer that could suggest a direct or indirect effect of vincristine treatment on the PMCs. Indeed, the PMCs are contractile cells and will therefore contain tubulin microtubules that may be targeted by vincristine treatment. The aim of future studies would therefore be to assess the location and function of the Sertoli cells and PMCs following treatment, as well as analysing the microtubules within these cells which are potentially vulnerable to damage by vincristine and have not been examined within this study.

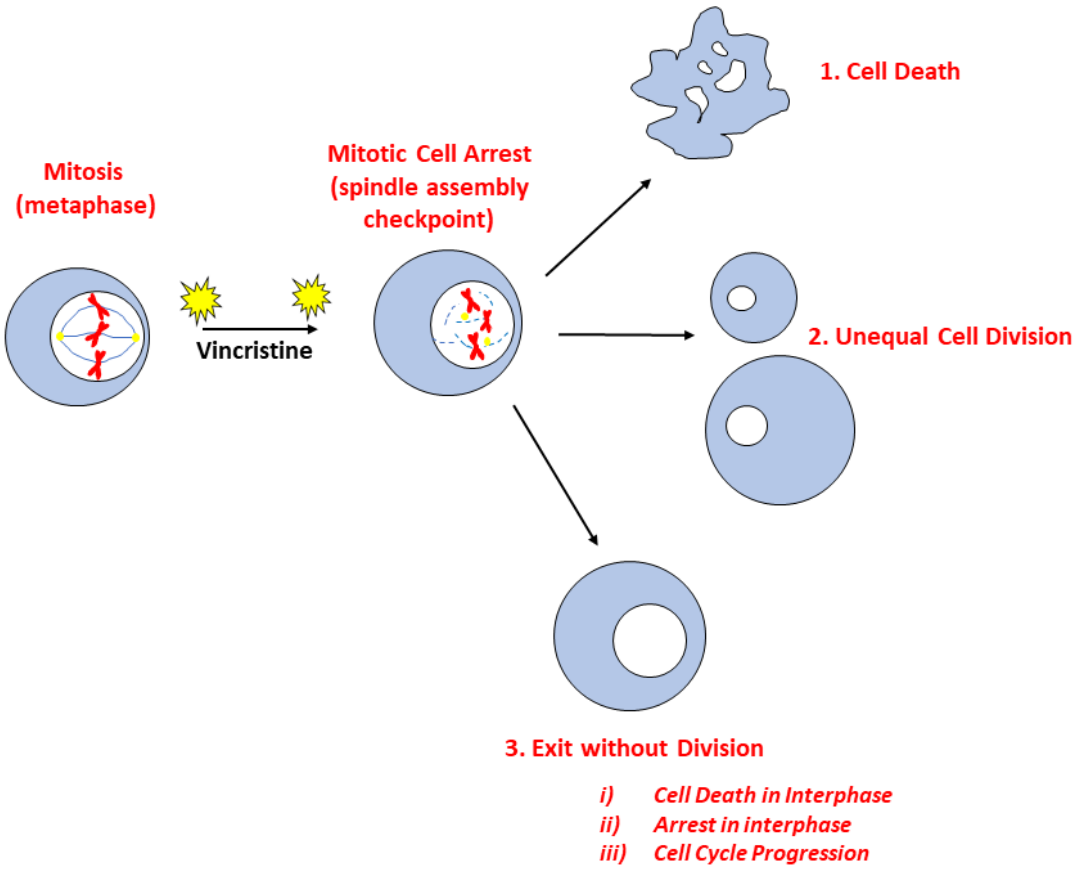


Figure 4.10: Potential fates of cells exposed to vincristine. Vinca alkaloids are classically considered anti-mitotic drugs where treatment results in mitotic arrest as cells fail to pass the spindle-assembly checkpoint. Cells can undergo either cell death in mitosis, unequal cell division or exit the cell cycle without undergoing cell division. Of those that exit without division; these cells can either undergo cell death during interphase, arrest during interphase or undergo additional cell cycles without dividing. Modified from Gascoigne and Taylor, (2009)

4.4.3 Relevance of results to patient serum levels

The concentrations of vincristine tested within this study were chosen to cover a large range that would also include patient serum levels that have been noted in clinical studies. Comparing, however, clinical patient serum levels and *in vitro* culture concentrations is difficult and will be discussed in detail in Chapter 7 (7.3.1). The effects of vincristine on the germ cell population within this study were observed at 0.1, 1 and 10µg/ml; with these concentrations resulting in effects *in vitro* that may

fall within clinical serum levels, with the higher limits of plasma levels of this drug found below 0.5µg/ml (steady state concentrations: 0.001-0.005µg/ml; Crom *et al.*, 1994; Kellie *et al.*, 2004). The serum levels stated here are from studies that have assessed, through differing methodologies, the concentration of vincristine within the plasma, amongst which multiple sources state that clinically relevant serum levels fall within the sub-micromolar range (reviewed in Jordan and Wilson, 2004; Dumontet and Jordan, 2010; Martino *et al.*, 2018). The concentration within cells, however, can be much higher than extracellular concentrations (in the 100-fold range), which have been noted in studies which have compared intra- and extracellular concentrations in cultures of HeLa cells (Lengsfeld *et al.*, 1982; Jordan *et al.*, 1991). The exact concentrations of vincristine to which the testis is exposed to and the concentrations within each cell are unknown and cannot be determined from assessment from plasma serum levels. Indeed, different cells have different sensitivities to the vinca alkaloids which may be due to differences in tubulin isomer (reviewed in Martino *et al.*, 2018).

A dose-dependent effect on the mechanism of action of vincristine has been shown *in vitro* with purified tubulin, low doses inhibited polymerisation of heterodimer tubulin (steady state of microtubule mass) compared to higher concentrations which induced active depolymerisation (decrease in microtubule mass) (reviewed in Dumontet and Jordan, 2010). This could also account for the differences noted in the germ cells following vincristine treatment over the large concentration range that were used in our study, from potentially 'low' concentrations (0.0001, 0.001 and 0.01µg/ml) to 'higher' doses (0.1, 1 and 10µg/ml) of vincristine. This switch in mechanism appears to occur over a narrow range of concentrations, for example a study in HeLa cells found 2.2 – 2.7nM vincristine inhibited mitosis, whereas a concentration of 9nM decreased microtubule mass indicative of active depolymerisation (Jordan *et al.*, 1991). The concentration switch from relative 'low' to 'high' concentration-dependent mechanism of action is most likely tissue/cell-specific, but is one possible explanation for our observations. Further studies should

include the visualisation of the microtubules to assess at which concentration this 'switch' occurs and to fully appreciate the underlying mechanism of action of vincristine on the prepubertal testis. This could include immunohistochemistry with use of anti-β-tubulin primary antibody to visualise the microtubules and/or techniques to assess microtubule dynamics (reviewed in Zwetsloot *et al.*, 2018).

4.4.4 Long-term recovery from vincristine treatment

As discussed above, the results of short-term cultures produced surprising results, with the accumulation of germ cells within the centre of seminiferous tubules, where these cells had not undergone cell death two-days post-exposure. These findings raised questions as to the longer-term fate of these germ cells, and whether there was potential for survival of the germ cells/SSCs, that would be essential for the prepubertal testis to recover from chemotherapy-induced damage and produce functioning spermatozoa in the adult. Investigations were therefore undertaken to determine the fate of the germ cell population, and in particular the SSCs, following a longer period of recovery (twelve-day recovery compared to two-day recovery). Initially tissues from 'n'=4 were collected, however, due to Covid-19, completion of the work to generate a sufficient 'n' to statistically analyse this pilot data was not possible. However, from the 'n'=2 analysed, there appeared to be a decrease in the density of the proliferating germ cells and the SSCs following vincristine treatment in both the short (four-day) and long (fourteen-day) term cultures, with notable clumping of cells in the short-term cultures, but absence of germ cells in the centre following the long-term cultures. This would therefore suggest that the cells within the centre of the seminiferous tubules have undergone cell death by twelve days post-treatment. As previously discussed, the germ cells present within the short-term cultures may represent germ cells that have undergone mitotic arrest, lost connection with the basement membrane and/or Sertoli cells and have therefore migrated into the centre of the seminiferous tubule. These germ cells consequently may have undergone cell death during interphase or subsequent cell cycles that take places between day two and day twelve post-treatment, resulting in the loss of these

clumps in the long-term cultures. Few germ cells did survive treatment with vincristine at the concentrations chosen for this study (0.05 and 0.1µg/ml) in the long-term, however, the occurrence of at least some SSCs along the basement membrane twelve days after treatment does show that there can be survival of at least some of this germ cell population, which could lead to repopulation with longer culture periods.

4.4.5 Why does our data differ from the clinical findings of vincristine-induced gonadotoxicity?

From this study, it is clear that vincristine treatment of the prepubertal testis at the concentrations tested effects the germ cell population, despite this drug clinically being considered safe to use in regards to later fertility (reviewed inWyns *et al.*, 2010). There are multiple explanations as to why our data differs from the clinical findings. The concentrations tested within our study may be higher than those found within the patient serum levels following treatment; however, it is difficult to compare serum levels to *in vitro* concentrations. The initial damage noted after two-days post-treatment of the prepubertal testis may be repaired through regeneration of the germ cell population resulting in normal fertility rates by adulthood. Indeed, our longer term cultures where damage was assessed twelve days post-exposure indicates there is potential for recovery with survival of few SSCs within the SSC niche. Cultures for longer periods of time following acute exposure, for example up to eight weeks as performed by Sato *et al.*, (2011b, 2011a) to determine whether the germ cells can undergo complete spermatogenesis would provide further evidence for this hypothesis. Our findings may be a result of an *in vitro* artefact; therefore, further *in vivo* studies with animal models should take place to confirm our results. Indeed, further *in vitro* studies with human tissue would be invaluable to determine whether the effects observed here with mouse tissue are species-specific. Lastly, the clinical studies which have deemed this drug as a 'low' gonadotoxic agent are often taken from studies where patients received a combination of chemotherapeutic agents, which may obscure the effects of individual drugs. This is why research using *in vitro*

and *in vivo* models to determine the gonadotoxicity of individual agents is essential to this field of research.

4.4.6 Conclusions

Despite the hypothesis that vincristine would have no significant impact upon the prepubertal testis, given that this drug is currently considered a 'low' gonadotoxic agent, the results found in this study clearly show that vincristine is gonadotoxic in an *in vitro* model within the range of concentrations assessed. Dose-dependent effects of vincristine treatment were observed with our prepubertal mouse testis culture model following exposure to vincristine for 24hrs with concentrations above 0.1µg/ml. Vincristine treatment resulted in damage to the testis with decreased germ cell density and number of proliferating germ cells, with accumulation of germ cells in the centre of the seminiferous tubules. These centralised germ cells were no longer apparent in long-term cultures, indicating that these germ cells had undergone cell death in the intervening period. Nonetheless, even after long-term cultures, there was survival of some SSCs within the SSC niche, highlighting the potential of this tissue for regeneration of the germ cell population following treatment: this could account for the normal fertility noted in clinical studies that include vincristine in treatment regimens. The impact of vincristine upon prepubertal mouse ovaries has also been assessed within our laboratory (see 3.2 for ovary culture). Dose-dependent effects were observed, with vincristine reducing the number of growing follicles and affecting the health of all follicles classes within the prepubertal ovary (Chloe Cairns, Caroline Allen, Vlastimil Srsen, Norah Spears; unpublished data). The dose-response for vincristine-induced damage will be used in additional chapters to assess the role of oxidative stress in chemotherapy-induced damage to the prepubertal testis.

Chapter 5. Role of ROS and Oxidative Stress in Chemotherapy-induced Damage to Mouse Prepubertal Testis and Rat GC-6spg Cell Line

5.1 Introduction

Understanding the underlying mechanism of action of chemotherapeutic agents is essential to aid development of cytoprotective strategies to protect healthy tissues from unwarranted damage as a side effect of treatment. The current, well-recognised mechanisms of action of chemotherapy drugs are based on studies using cancerous cells/tumours, with research on healthy tissues to detect the molecular targets of these drugs performed when severe toxicity to specific organs has been observed (see Chapter 1, Table 1.1 for mechanisms of action of classes of chemotherapy drugs). A full understanding of the molecular targets of these drugs is not entirely understood, even less so when considering the damage induced in healthy tissues: as such, it requires continued research.

Considering the prepubertal testis tissue, there are very few studies into the mechanism of action of drugs: research into gonadotoxicity on this tissue is rare, current gonadotoxicity classifications are debated and knowledge of the molecular targets in the testis are usually based on adult studies. This chapter will explore the role of oxidative stress in cisplatin and doxorubicin-induced damage to the prepubertal mouse testis and in a rat GC-6spg cell line, representative of SSCs. A role for ROS and oxidative has been described in relation to known toxicities of these drugs, including cisplatin-induced nephrotoxicity and doxorubicin-induced cardiotoxicity (reviewed in Chen *et al.*, 2007); however the role in gonadotoxicity is unclear. Cisplatin is a chemotherapy agent from the platinum containing alkylating-like drug class, which was first synthesised in the 1840s and FDA approved as a chemotherapy agent in the late 1970s (reviewed in Dasari and Tchounwou, 2014). Doxorubicin is a chemotherapy agent of the anthracyline drug class, and was originally isolated from *Streptomyces peucetius caesius* fungus in the early 1970s (reviewed in Thorn *et al.*, 2011). In addition, the chemotherapy drug vincristine is included in these studies to act as a 'negative' control drug since currently vincristine

has not been considered to induce high levels of oxidative stress as a mechanism of toxicity (reviewed in Conklin, 2004) .

5.1.1 Reactive Species and Oxidative Stress

Oxidative (or nitrosative) stress results from an imbalance in reactive oxygen or nitrogen species (ROS/RNS) production and antioxidant defence mechanisms that can cause higher than normal levels of ROS/RNS, resulting in disruption to redox signalling and/or cellular damage (reviewed in Birben *et al.*, 2012; Sies, 2015). ROS/RNS include free radicals that are chemical species with unpaired electrons, which increases the chemical reactivity of the molecule/atom until it accepts (reduction) or donates (oxidation) an electron to another species to achieve stability (oxidation-reduction, redox reaction) (reviewed in Cheeseman and Slater, 1993; Betteridge, 2000). Examples of free radicals include hydroxyl radicals ($OH^{\bullet}$), superoxide radicals ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$). ROS/RNS can also include non-radicals that are oxidants, for example hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and peroxynitrite (ONOO-), which are involved in the production of key free radicals (reviewed in Genestra, 2007; Phaniendra *et al.*, 2015) (examples of ROS/RNS shown in Table 5.1). Free radicals are produced as a by-product of many important cellular processes including aerobic respiration (accidental release from the electron transport chain) or during phagocytosis (deliberate release for microbial defence by action of nicotine adenine dinucleotide phosphate [NADPH] oxidases). Free radicals can also be produced from exogenous sources, for example air pollutants or certain drugs (reviewed in Cheeseman and Slater, 1993; Betteridge, 2000; Pham-Huy *et al.*, 2008; Birben *et al.*, 2012) (see Table 5.1 for further examples). Often, the production of ROS/RNS is self-sustaining, with redox cycling amplifying the production of these ROS/RNS. The most abundant source of free radicals within cells is through the leakage of electrons from the electron transport chain in the inner membrane of the mitochondria during oxidative phosphorylation and ATP production. Around 2% of electrons are accidentally released during aerobic respiration: these react with oxygen to form the superoxide radical ($O_2^{\bullet-}$) (reviewed in Cheeseman and Slater,

1993; Conklin, 2004). The superoxide radical ($O_2^{\bullet-}$), rather than inducing significant amounts of damage to cells, leads to the formation of further ROS/RNS species, for example generating hydrogen peroxide (H_2O_2) and the most potent ROS, the hydroxyl radical ($OH^{\bullet}$) (Fenton and Haber-Weiss reactions, reaction with transition metals ions) (see Fig 5.1A for overview). In the testis, ROS can be produced through the action of enzymes including NADPH oxidases, xanthine oxidases as well as through the action of cytochrome P450 enzymes in steroidogenic tissues (Bánfi *et al.*, 2001; Kumagai *et al.*, 2002; reviewed in Hanukoglu, 2006).

Table 5.1: Overview of ROS/RNS as well as examples of endogenous and exogenous sources. Information derived from Phaniendra *et al.*, (2015)

Reactive Oxygen and Nitrogen Species	Internal Sources	External Sources
Superoxide Radical ($O_2^{\bullet-}$)	Mitochondria	Cigarette smoke
Hydroxyl Radical ($OH^{\bullet}$)	Inflammation	Environmental pollutants
Hydrogen Peroxide (H_2O_2)	Phagocytosis	Radiation
Singlet Oxygen ($^1O_2$)	Arachidonate Pathway	Certain drugs e.g. pesticides
Alkoxyl/Peroxyl Radicals ($RO^{\bullet}/ROO^{\bullet}$)	Exercise	Industrial Solvents
Peroxynitrite ($ONOO^-$)	Ischemia/Perfusion Injury	Ozone
Nitric Oxide ($NO^{\bullet}$)	Xanthine Oxidase	High Temperature
Nitrogen Dioxide ($NO_2^{\bullet}$)	Peroxisomes	Alcohol
Transition Metals Iron & Copper	NADPH oxidase complex	Cooking (e.g. smoked meats)
	Cytochrome P450	
	Endoplasmic Reticulum	

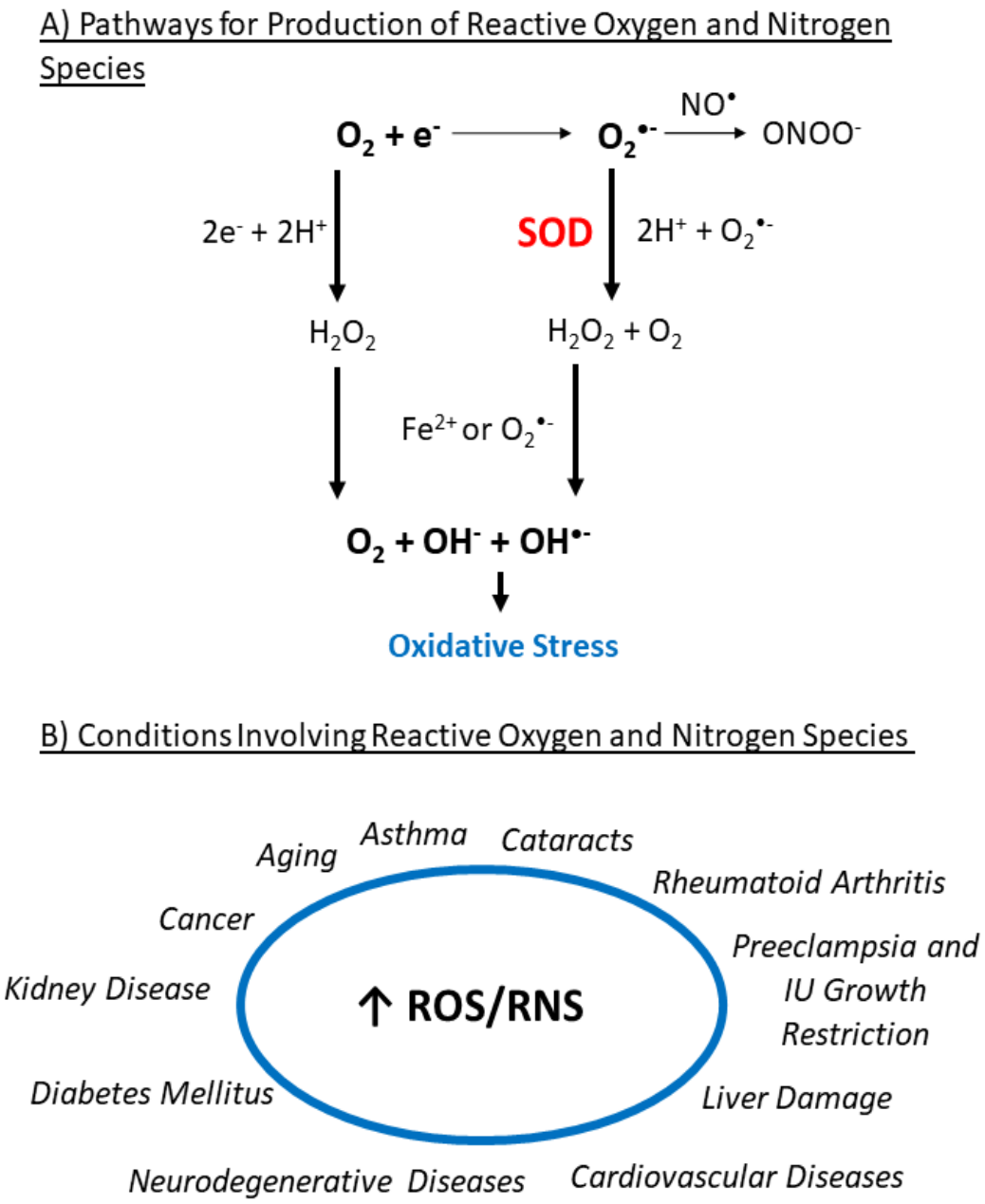


Figure 5.1: Overview of chemical reactions leading to ROS production and medical conditions where a role of ROS has been described. A) ROS generation through electron transfer results in production of superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). The superoxide radical can be converted into hydrogen peroxide (H_2O_2) and oxygen (O_2) through action of superoxide dismutase enzyme (SOD). Peroxynitrite radical (ONOO-) is also produced upon reaction of nitric oxide (NO) with the superoxide radical ($O_2^{\bullet-}$). Modifiefed from Devine *et al.* (2012). B) Medical conditions/diseases with ROS involvement. Information derived from Cheeseman and Slater, (1993) and Phaniendra *et al.* (2015). IU; intrauterine.

The production of free radicals has been implicated in numerous human diseases, including cancer, as shown in Fig 5.1B (reviewed in Pham-Huy *et al.*, 2008; Lobo *et al.*, 2010; Phaniendra *et al.*, 2015). In addition, free radicals are essential for normal functioning of cells and have been shown to act as a signalling molecules in multiple pathways involved in cell survival and proliferation (MAP kinases, PI3 kinases, PTEN and protein tyrosine phosphatases) (reviewed in Ray *et al.*, 2012). For example, hydrogen peroxide (H_2O_2) can signal the activation of a range of transcription factors including AP-1, NRF2 and NK–κB (Marinho *et al.*, 2014). A role for free radicals in stimulating the activation and execution of apoptosis has also been explored, with ROS investigated as a stimulator of apoptosis (reviewed in Kannan and Jain, 2000; Carmody and Cotter, 2001; Ozben, 2007). Assessment of ROS/RNS production is difficult due to the transient nature of these compounds that are often highly reactive, unstable and short-lived (reviewed in Betteridge, 2000). Studies have therefore focused upon the resulting oxidative/nitrosative stress-induced damage that is produced because of ROS/RNS production. Antioxidants are defence mechanisms against the free radicals produced by the cell that maintain the balance to prevent oxidative or nitrosative stress; this will be discussed in more detail in Chapter 6.

The overproduction of ROS/RNS can lead to oxidative or nitrosative stress; these highly reactive species can react with biological macromolecules within a cell, including lipids, proteins, nucleic acids or carbohydrates (reviewed in Betteridge, 2000; Phaniendra *et al.*, 2015). ROS/RNS can damage nucleic acids of DNA, with mitochondrial DNA more susceptible than nuclear DNA due to the production of free radicals during aerobic respiration (reviewed in Phaniendra *et al.*, 2015). RNA is also highly susceptible to free radical damage due to its single strand nature and due to the lack of repair mechanisms (Hofer *et al.*, 2005). ROS can react with all components of nucleic acids including the bases and backbone, inducing single and double strand breaks. Interactions of the hydroxyl radical with RNA/DNA bases can result in a range of different adducts when attacking pyrimidine (e.g. thymine/uracil glycol) or purine

(e.g. 8-hydroxydeoxy guanosine, 8-OHdG) bases, as well as the sugar backbone (e.g. glycolic acid) (reviewed in Dizdaroglu *et al.*, 2002). Additionally, RNS such as peroxynitrite (ONOO-) can interact with guanine bases leading to the formation of 8-nitroguanine and 8-oxodeoxyguanosine lesions (Yermilov *et al.*, 1995). The ROS/RNS species can attack polyunsaturated fatty acids such as those of phospholipid membranes, which can lead to membrane dysfunction including reduced fluidity and inactivation of membrane receptors and enzymes. This process is known as lipid peroxidation, where free radicals remove a hydrogen atom from the fatty acid, resulting in the formation of a radical with a carbon atom and a free electron. This radical undergoes molecular rearrangement, reacts with oxygen and forms the lipid peroxyl radical ($ROO^{\bullet}$) which can further stimulate lipid peroxidation and result in formation of lipid hydroperoxides (reviewed in Betteridge, 2000). The lipid hydroperoxides are unstable and decompose to form reactive aldehyde end-products, including thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA) and 4-hyroxylnonenal (HNE), which are stable products that can diffuse into the cell and cause further damage, particularly to proteins (reviewed in Lobo *et al.*, 2010; Birben *et al.*, 2012). Proteins are susceptible to ROS/RNS attack that result in modifications of amino acids, peptide cleavage or cross-linkage of proteins, resulting in proteolysis. Amino acid modifications include formation of protein carbonyls. Amino acids that contain sulphur (thiols) are particularly susceptible, including methionine and cysteine that are converted to disulphide and methionine sulfoxide. The resulting damage to proteins can result in a wide range of cellular dysfunction including loss of function of enzymes and receptors (reviewed in Butterfield *et al.*, 1998; Lobo *et al.*, 2010; Phaniendra *et al.*, 2015). A common marker of protein damage is nitrotyrosine that is a product of RNS attack on tyrosine residues: this marker along with 8-OHdG (DNA/RNA damage) and MDA (lipid peroxidation) are used in this thesis to assess for oxidative stress in mouse prepubertal testicular tissue following cisplatin, doxorubicin or vincristine treatment.

5.1.2 Oxidative stress in the testis

The testis is susceptible to oxidative stress, which has been linked to male infertility, along with a range of conditions inducing oxidative stress including cryptorchidism, testicular torsion and varicocele (reviewed in Aitken and Roman, 2008; Turner and Lysiak, 2008). Most studies, however, that have looked at the relation between oxidative stress and gonadotoxicity have focused upon the adult testis and on spermatozoa, which are vulnerable to oxidative stress due the their morphology (reviewed in Turner and Lysiak, 2008; Mortimer *et al.*, 2013; Asadi *et al.*, 2017; Aitken, 2020). It is accepted that testis tissues are susceptible to lipid peroxidation in particular, due to the high content level of polyunsaturated fatty acids, this however may be specific to more differentiated spermatogonia and spermatozoa (reviewed in Aitken and Roman, 2008; Turner and Lysiak, 2008). Indeed, during spermatogenesis the lipid composition and volume of the germ cell membrane changes with maturation, resulting in high levels of polyunsaturated fatty acids in the sperm membrane that aid in sperm motility, acrosomal reaction and sperm-oocyte fusion (reviewed in Collodel *et al.*, 2020). To date, there have been few studies into oxidative stress in the prepubertal testis. The adult testis is susceptible to ROS attack due to low oxygen tension and high-energy demands of spermatogenesis and Leydig cell steroidogenesis, which can lead to increased production of free radicals from the mitochondria (Free *et al.*, 1976; reviewed in Aitken and Roman, 2008). This generalisation may not apply to the prepubertal testis since this tissue is not undergoing active spermatogenesis or steroidogenesis, and generally has a reduced proliferation rates compared to the adult testis (Müller and Skakkebaek, 1983).

5.1.3 Oxidative Stress in Cancer and Chemotherapy-Induced Damage

5.1.3.1 Cancer

The role of ROS/RNS in cancer is an area of research that is controversial, as studies have found ROS/RNS to be involved in the promotion, progression and inhibition of tumorigenesis (overview in Fig 5.2) (reviewed in Barrera, 2012; Hayes *et al.*, 2020). Cancer cells/tumors have higher levels of ROS/RNS production and oxidative stress

than normal cells/tissues, this is as a result of altered cellular metabolism to support growth and survival of the cancer (reviewed in Cairns *et al.*, 2011). ROS/RNS at low levels have a role in stimulating proliferation and promoting cell survival and as discussed above, can induce damage to DNA, which can lead to mutagenesis. For example, the 8-OHdG adduct can result in transverse mutations from GC-TA forming transformed cells. Indeed high levels of 8-OHdG have been found in certain cancerous tissues (Kubo *et al.*, 2014; reviewed in Chikara *et al.*, 2018). Moderate levels of ROS can support expansion of transformed cells by inducing production of pro-survival signalling factors such as glucose transporters and vascular endothelial growth factor, through upregulation of hypoxia inducible factor (reviewed in Bell *et al.*, 2005; Cairns *et al.*, 2011). High levels of ROS/RNS, alternatively, can lead to cell senescence where ROS are produced through action of the tumour suppressor proteins, pRB (retinoblastoma) and p53. This leads to activation of protein kinase C δ which blocks cytokinesis and maintains normal cells in senescent state (Ramsey and Sharpless, 2006; Takahashi *et al.*, 2006). In addition, high levels of ROS/RNS can lead to cell death through apoptosis or necrosis, depending upon the levels of reactive species produced (reviewed in Carmody and Cotter, 2001; Ozben, 2007). In regards to apoptosis, ROS/RNS can increase the permeability of the outer membrane of mitochondria, leading to the release of cytochrome C. Cytochrome C is heavily involved in the activation of the intrinsic apoptotic pathway leading to the activation of initiator caspases such as caspase 3 and 9, ultimately resulting in programmed cell death (reviewed in Garrido *et al.*, 2006). There is therefore an important balancing act taking place within cancer cells to maintain tumor growth without stimulating cell senescence or death, this is achieved through upregulation of antioxidants (reviewed in Gorrini *et al.*, 2013) (Fig 5.2).

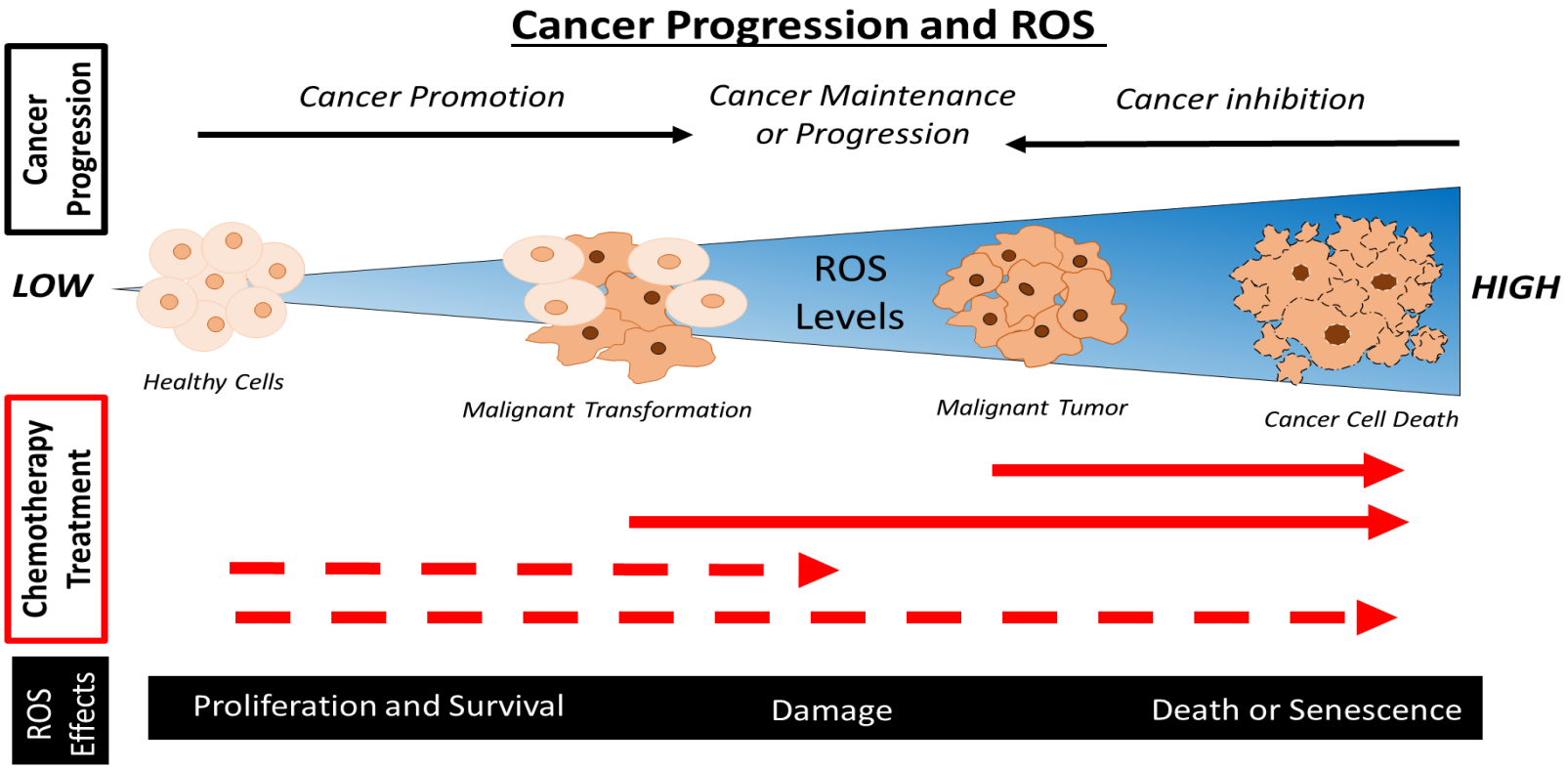


Figure 5.2 Reactive oxygen species (ROS) in cancer and anti-cancer therapy. Differing effects of ROS in cancer progression (low ROS levels supports proliferation and cell survival whereas high levels induces cell death and senescence). Changes in cancer metabolism and protein translation result in increased ROS production leading to cancer promotion, whereas antioxidants prevent high levels of ROS production to inhibit cancer. Cancer cells tightly regulate ROS production and antioxidants to maintain a balance to support cancer growth. The use of chemotherapy drugs which increases ROS levels during cancer progression can result in cancerous cells undergoing cell death. Chemotherpy treatment can also impact on healthy cells resulting in cell damage or death. Image modified from Cairns *et al.* (2011); Gorrini *et al.* (2013).

5.1.3.2 Chemotherapy Treatment

Anti-cancer treatments that cause excessive levels of ROS/RNS and oxidative stress could be employed to overcome the antioxidant defence mechanisms of cancer cells, pushing them towards cell death (reviewed in Gorrini *et al.*, 2013) (Fig 5.2). Certain chemotherapy drugs are known to induce oxidative stress, with anthracyclines (doxorubicin) and platinum containing alkylating-like agents (cisplatin) considered to induce high levels and vinca alkaloids (vincristine) low levels (Fig 5.3) (reviewed in Conklin, 2004). Increased ROS following chemotherapy treatment can be a result of overproduction of ROS, for example through targeting the mitochondria, or by interfering with antioxidant systems. Studies comparing plasma of patients before and after chemotherapy, or comparing treated to untreated, have found enhanced levels of lipid peroxidation products (MDA, TBARS) and decreased antioxidants levels (Sangeetha *et al.*, 1990; Erhola *et al.*, 1996; Faure *et al.*, 1996; Weijl *et al.*, 1998; Jonas *et al.*, 2000; Cetin *et al.*, 2004; Conklin, 2004; Nowak and Janczak, 2006; Santiago-Arteche *et al.*, 2012). Overall, this is a simplistic view of the role of oxidative stress in chemotherapy-induced damage; other studies have found adverse effects of oxidative stress with anti-cancer treatments (reviewed in Conklin, 2004). Increased oxidative stress, which can result in enhanced levels of reactive aldehydes (lipid peroxidation), may halt cell cycle progression at the G0 to G1 phase causing cell cycle arrest at checkpoints (reviewed in Conklin, 2004). For example, investigations in cancer cell lines have shown increased 4-HNE levels reduce cellular proliferation through increased expression of p53, a protein which controls cell cycle progression (Laurora *et al.*, 2005; Barrera, 2012). This would interfere with the ability of certain chemotherapy agents that are cell cycle specific in their mechanism of action and therefore reduce chemotherapeutic effectiveness (reviewed in Conklin, 2004). In addition, reactive species can reduce chemotherapy-induced apoptosis. In the Shacter *et al.* (2000) study, hydrogen peroxide (H_2O_2) treatment in lymphoma cells shifted the mode of cell death of chemotherapy agents from apoptosis to necrosis. Apoptosis is a more 'advantageous' mode of cell death, since apoptotic bodies are phagocytosed whereas in necrosis, cell membrane integrity is lost and the contents

of cells released, which can induce an inflammatory response. Additional studies have shown that ROS/RNS can target the protein caspases and reduce activity of these key effectors of apoptosis, for example upon hydrogen peroxide treatment (Hampton and Orrenius, 1997; reviewed in Conklin, 2004).

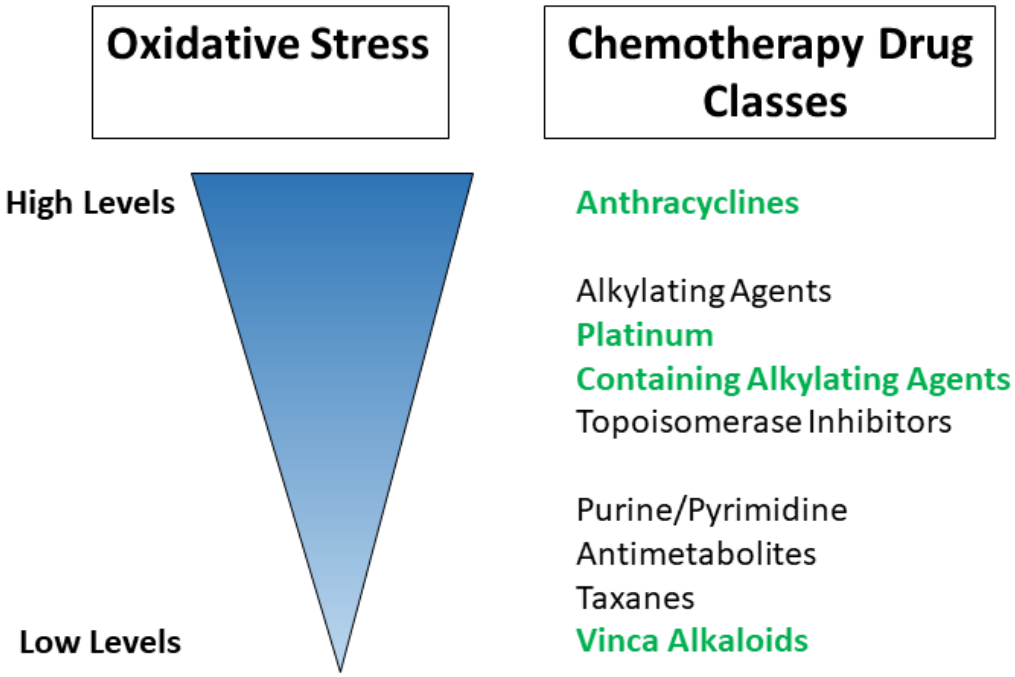


Figure 5.3: Classification of chemotherapy agents depending upon levels of oxidative stress produced. Green highlights the chemotherapy drug classes from which the drugs investigated in this PhD study belong too. Modified from Conklin, (2004)**.**

Due to the production of ROS/RNS upon chemotherapy treatment, oxidative stress can be induced in non-targeted tissues, a process that can result in toxic side effects. Inducing oxidative stress in normal cells can potentially lead to cellular damage or cell death (Fig 5.2). As a result there can be reduced chemotherapy usage, or a decrease in the quality of life for cancer survivors (reviewed in Chen *et al.*, 2007). Chemoprotective strategies for healthy tissues are therefore required to protect against these toxic side effects, without limiting the anti-cancer action of chemotherapy agents. Oxidative stress is currently known to be involved in certain toxicities of chemotherapy drugs that induce high levels of oxidative stress.

Cardiotoxicity, nephrotoxicity and damage to the brain following doxorubicin treatment has been partly attributed to oxidative stress (Lahoti *et al.*, 2012; Keeney *et al.*, 2018; reviewed in Songbo *et al.*, 2019). The production of ROS and oxidative stress in cardiac tissues is complex, and thought to involve multiple pathways/mechanisms. The redox cycling of the quinone sugar moiety in the tetracycline ring of doxorubicin to semiquinone releases ROS. In addition, free radicals can also be produced due to mitochondrial dysfunction, doxorubicin acting as an iron chelator, formation of 7-deoxydoxorubicinone upon cleavage of the glyosidic bond, reduction in antioxidant levels and due to disruption in nitric oxide regulation (reviewed in Minotti *et al.*, 2004; Thorn *et al.*, 2012; Songbo *et al.*, 2019). Nephrotoxicity following cisplatin treatment has also been linked to oxidative stress (reviewed in Volarevic *et al.*, 2019). Cisplatin induces ROS formation by targeting the mitochondria as the drug binds to mitochondrial DNA and proteins resulting in mitochondrial dysfunction due to diminished protein synthesis of electron transport chain proteins. In addition, cisplatin can inhibit calcium uptake as well as reducing mitochondrial membrane potential (Marullo *et al.*, 2013; reviewed in Dasari and Tchounwou, 2014). Whether the underlying mechanism of actions that currently include oxidative stress in their mode of toxicity are tissue specific is unknown, with the role of oxidative stress in reproductive tissues requiring further research.

5.1.4 Aims and Hypothesis

Based on the known role of oxidative stress in both cisplatin and doxorubicin-induced toxicity, it was hypothesised that free radicals and oxidative stress are involved in cisplatin- and doxorubicin-induced gonadotoxicity. In regards to vincristine, due to the proposed lower levels of oxidative stress induced upon treatment, it was hypothesised that ROS/RNS species and oxidative stress would not be a part of the underlying mechanism of action of this drug. This chapter therefore set out to examine levels of oxidative stress in the prepubertal mouse testis and production of ROS in the GC-6spg cell line following chemotherapy treatment, in a time-dependent manner.

5.2 Methods

5.2.1 Role of oxidative stress in chemotherapy-induced damage to the prepubertal mouse testis

To examine the role of oxidative stress in chemotherapy-induced damage to the prepubertal testis, a dose-response for cisplatin and doxorubicin was required to determine concentrations that induce substantial germ cell loss, in order to work out doses to then use in the time-course study to assess for the presence of oxidative stress. A dose-response for vincristine was already determined in Chapter 4 (see 4.3.1), with results from that Chapter used here to select the concentration of vincristine in the oxidative stress time-course study.

5.2.1.1 Experiment 1: Confirming dose-response for cisplatin and doxorubicin on density of germ cells in prepubertal mouse testis

A dose-response for cisplatin was initially determined in Chapter 3 (see 3.2) to compare cisplatin and carboplatin, where ~0.1mm^3 testis fragments were exposed to cisplatin prepared from powder and cultured at 37^{o}C (see Chapter 3.3.2). For further studies into the mechanism of action of the chemotherapy drugs, larger fragments of 1mm^3 were cultured to increase the number of sections collected from each testis fragment (see 2.1.2), and a clinical preparation of cisplatin used (see 2.2). Cultures were therefore undertaken to determine a new dose-response for cisplatin by exposing 1mm^3 testis fragments to a range of concentrations of clinical preparation of cisplatin (0.01, 0.05, 0.1, 0.5, 1, 5 or 10µg/ml) for 24hrs on day two of culture. In addition, a dose-response for doxorubicin was determined where testis fragments were exposed to 0.005, 0.05 or 0.5µg/ml doxorubicin for 24hrs on day two of culture. The excipients for the clinical preparations of cisplatin and doxorubicin include sodium chloride and sodium hydroxide/hydrochloric acid for pH adjustment as well as water for injections. Control tissues for cultures using clinical preparations of the drugs were exposed to equivalent volumes of PBS. A sample size of 'n'=3 from three cultures for cisplatin-exposed tissues and 'n'=2 from two cultures for doxorubicin-exposed tissues, using clinical preparations of the drugs. To rule out the potential

effects of the excipients, additional cultures were run with the powdered form of doxorubicin made up in distilled water with control tissues exposed to equivalent volumes of water ('n'=4, from four culture runs). Cultures with the powdered form of doxorubicin were performed by myself, with the tissues microtomed and immunofluorescence performed by student Eva Baxevanidi under my supervision. Analysis for all cultures was undertaken by myself, where the density of the germ cells and proliferative capacity of these cells analysed (MVH-positive, BrdU-positive or negative).

5.2.1.2 Experiment 2: Time-course of expression of oxidative stress markers in prepubertal mouse testis

5.2.1.2.1 Culture

To determine the time-course of oxidative stress following chemotherapy treatment of the prepubertal testis, tissues were fixed at selected time points during culture, at 6, 16, 24, 30, 48 and 72hrs post-exposure to the chemotherapy agents. These timings were selected to collect tissues during the 24hr window of drug exposure (6, 16 and 24hr time points) and to cover the two-day recovery period (30, 48 and 72hrs). The tissues fixed at the 72hr time point are equivalent to the tissues fixed at the end of the four-day culture period used for initial dose-response cultures. The dose-response for cisplatin, doxorubicin and vincristine (see 4.3.1) were used to select concentrations of each drug that induced considerable loss of the germ cell population. Time-course cultures were set up as described in Chapter 2 (see 2.1) with the tissues exposed to either 1µg/ml cisplatin, 0.1µg/ml doxorubicin or 0.5µg/ml vincristine. The clinical preparations of cisplatin and doxorubicin were used as these drugs were made up in sodium chloride, therefore PBS could be used for control tissues. The vincristine clinical preparation, as previously discussed, includes the excipient mannitol at unknown concentrations, therefore vincristine powder was used for these cultures with the powder prepared using PBS to enable a single control group for all three drugs investigated. Control and drug exposed tissues were collected at 6, 16, 24, 30, 48 and 72hrs post drug exposure, with the tissues fixed as

described in Chapter 2 (see 2.3.1). In addition, 800µl of media was collected at each time point and stored at -80^{o}C. Further studies were planned to test the media for antioxidant capacity following chemotherapy drug exposure using the total antioxidant capacity kit (Abcam, ab6532). Due to Covid-19, however, these experiments were not performed. Cultures were set up by myself, with a master's student, Emma Guy, performing the microtoming, immunofluorescence and analysis for an 'n'=3 (total 'n'=4 from two culture runs) under my day-to-day supervision.

5.2.1.2.2 Optimization of Oxidative Stress Antibodies

To determine whether the chemotherapy drugs cisplatin, doxorubicin and vincristine induced oxidative stress, immunofluorescence was used to assess for lipid peroxidation and DNA/RNA damage of the prepubertal testis tissue at the selected time points. The oxidative stress antibodies had not been used within the lab and were therefore optimized before use with experimental tissue. The primary/secondary antibodies and dilutions tested are shown in Table 5.1. A marker for 8-OHdG adducts was used to assess for DNA/RNA lesions that result from ROS damage. MDA marker was used to measure lipid peroxidation, since MDA is produced as a result of peroxidation of polyunsaturated fatty acids. The oxidative stress antibodies were tested and optimized using positive biological control back skin tissue, as suggested by Abcam since oxidative stress is apparent in the epidermis layer of skin (Fig 5.4A). Back skin tissue was harvested by Atanaska Velichkova (Carole Torsney lab) from 5-week old female C57BL/6JCrl (transgenic for FOS) mice. The tissue was cut into rectangular pieces and placed dermis side down onto Whatman filter paper; this ensured that during fixation the tissue remained flat. The tissue was fixed in 10% neutral buffered formalin for 2-3hrs and processed for wax embedding as described in Chapter 2 (2.3.1). The back skin tissue was microtomed along the long axis at 5µm for immunofluorescence imaging, where different dilutions of the antibodies were tested (Fig 5.4 i, ii, iii). In addition to the back skin, pnd four mouse ovaries from experiments investigating the impact of benzo[a]pyrene (B[a]P) on the female reproductive system were used as additional biological controls. These

experiments involved culturing ovaries as described in Chapter 3 (see 3.2) and exposing the tissues for 24 hrs to either 1µg/ml B[a]P (Sigma, B1760 dissolved in 0.1% DMSO) or to 0.1% DMSO as a control. These tissues were fixed after the 24hr exposure window, processed and microtomed as described in Chapter 2 (see 2.3). B[a]P experiments were performed primarily by Dr Agnes Stefansdottir and additionally by Emma Guy (masters student). B[a]P is a polycyclic aromatic hydrocarbon that is a component of cigarette smoke and has been shown by Dr Agnes Stefansdottir (personal communication/manuscript) and others (Zhang *et al.*, 2018) to induce oxidative stress in the ovary. Tissues from these experiments were therefore ideal to act as both a negative (DMSO-treated) and a positive (B[a]P-treated) biological control. An additional antibody against protein peroxidation, anti-nitrotyrsosine (Sigma, n0409) was also optimized but was not used on experimental tissue due to time constraints.

Table 5.2: Primary/secondary antibodies used within this chapter to assess for oxidative stress. This includes the dilution factors tested on backskin tissue and the final dilutions used.

Primary Antibody	Tested Primary Ab Dilutions	Final Primary Ab Dilution	Secondary Antibody (1:200)
Rabbit anti-MDA (Abcam, ab6463)	1:1000 1:2500 1:5000	1: 1000	Alexafluor goat anti-rabbit IgG 568 nm
Mouse anti-8-OHdG (Santa Cruz, SC-66036)	1:100 1:500 1:1000	1:100	Alexafluor goat anti-mouse $IgG_{2\beta}$ 488nm
Rabbit anti-nitrotyrosine (Sigma, n0409)	1:1000 1:2500 1:5000	Not included	Alexfluor goat anti-rabbit IgG 568nm

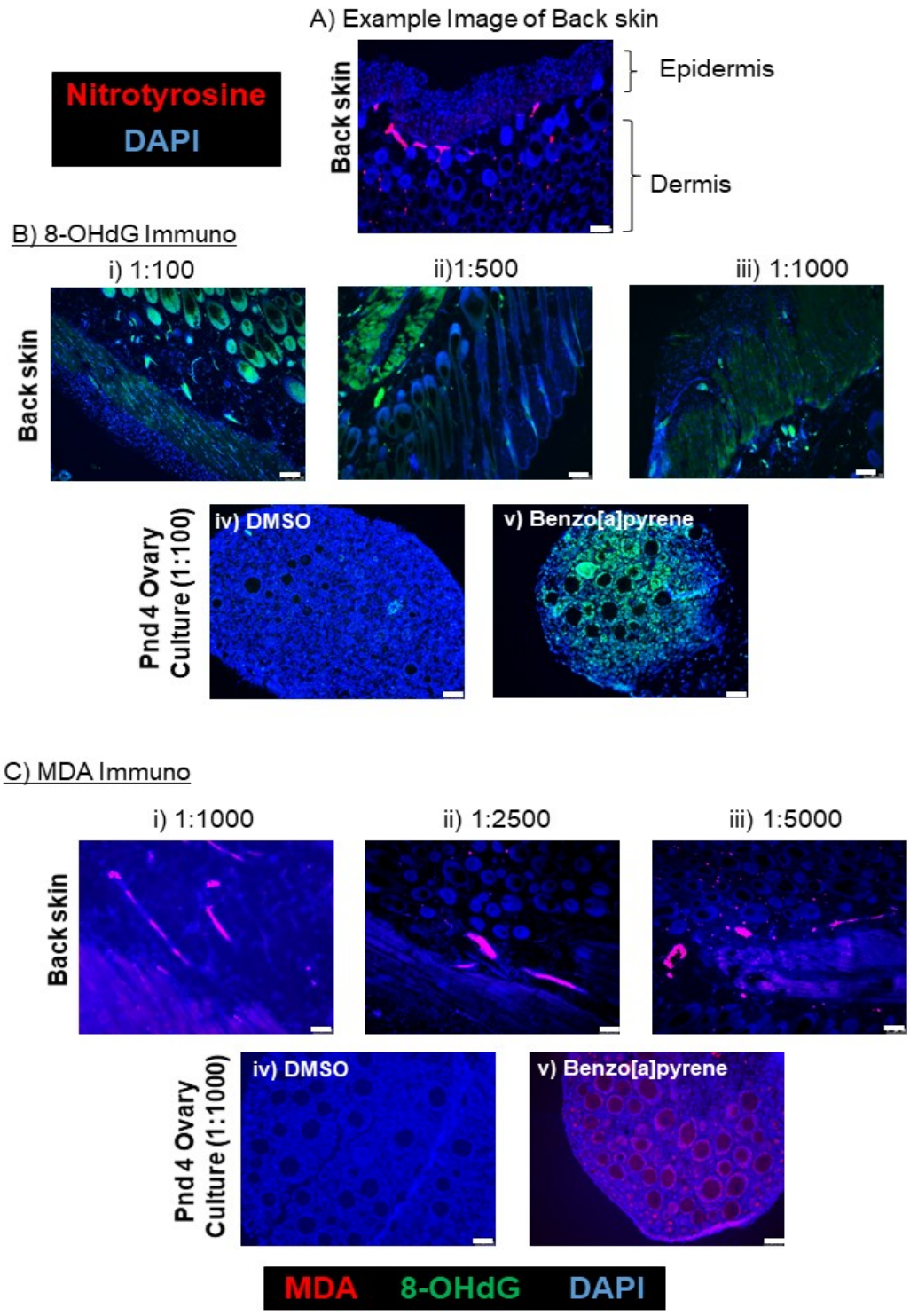


Figure 5.4 Optimization of 8-OHdG and MDA primary antibodies. (A) Biological positive control tissue suggested by Abcam includes back skin tissue where oxidative stress can be observed in the epidermal layer (red signal). (Bi, ii and iii) The 8-OHdG primary antibody and (Ci, ii and iii) MDA primary antibody was optimized for concentration on back skin tissue at differing concentrations. Positive and negative biological control tissues include (iv) DMSO and (v) benzo[a]pyrene exposed tissues.

5.2.1.2.3 Immunofluorescence

The time-course of oxidative stress following cisplatin-, vincristine- or doxorubicin-induced damage to the prepubertal testis was examined through immunofluorescence imaging of the prepubertal testis for lipid peroxidation and DNA/RNA damage at 6, 16, 24, 30, 48 and 72hrs post-exposure to the drugs. For an immunofluorescence experiment, a slide from the middle of each tissue from control, cisplatin, doxorubicin and vincristine treated tissues at each time point was included in an individual run. In addition, DMSO and B[a]P treated ovaries were included in each run to act as biological controls. Negative controls with the primary antibody omitted were also included for both cultured testis and ovary tissues. Double immunofluorescent imaging of MVH (Table 2.1) and either MDA (lipid peroxidation) or 8-OHdG (DNA/RNA damage) were used to assess the germ population and the presence of oxidative stress.

5.2.1.2.4 Analysis

The density of the germ cell population was assessed through manual counting of MVH positive cells per area of section due to expected loss of architecture following vincristine treatment which would make it difficult to measure seminiferous tubule area. 8-OHdG expression was examined through semi-automative analysis of area expression of oxidative stress markers per interstitial area of tissue (shown as percentage). The interstitial area was calculated by subtracting the seminiferous tubule area from the total area of tissue. This was performed due to the positive signal within the interstitium. I undertook analysis of germ cell density whereas Emma Guy performed the analysis of 8-OHdG expression under my supervision.

5.2.3 Role of ROS in chemotherapy-induced damage to the GC-6spg cell line

Oxidative stress markers were used to assess for damage in the prepubertal testis, however such markers do not allow for direct measurement of ROS production. This approach does not enable determination of whether the chemotherapy agents themselves induce ROS and therefore oxidative stress, or if the resulting cell death

produces the ROS. In order to address this question, the GC-6spg cell line was used along with a fluorescent probe, CellROX, to assess ROS production after chemotherapy exposure, as well as with a fluorescent probe to measure cell death, CellEVENT. The CellROX probe fluoresces upon production of ROS/RNS and could therefore be used, in conjunction with CellEVENT, to determine the timing of ROS production and cell death in cultures of GC-6spg cells following cisplatin, doxorubicin or vincristine treatment.

5.2.3.1 Experiment 3: Establishing a dose-response for cisplatin-, doxorubicin- and vincristine-induced cytotoxicity to GC-6spg cell line by assessing cell viability

The concentrations of chemotherapy drugs that reduce cell viability, as a measure of cytotoxicity, of the GC-6spg cell line was first determined by elucidating the dose-response for each drug following 48hrs of chemotherapy drug exposure. The GC-6spg cell line was cultured and exposed to chemotherapy drugs, with cell viability determined by MTT assay as described in chapter 2 (2.6). Clinical preparations of doxorubicin and cisplatin were utilized, whereas the powder form of vincristine dissolved in PBS was used. This enabled a single control group for these experiments, which were exposed to equivalent volumes of PBS as drug vehicle common to all three drugs. The dose-response relationship was determined for cisplatin 0.05-10µg/ml, doxorubicin 0.05-5µg/ml and vincristine 0.0001-10µg/ml. Based on the dose-response, concentrations of each drug that reduced cell viability by ~50% (known as the EC50 dose i.e. half-maximal effective drug concentration) was used for additional experiments.

5.2.3.2 Experiment 4: Time-course of CC3/7 expression following chemotherapy treatment of GC-6spg cell line

To determine the time-course for apoptosis, the CellEVENT probe was used to measure CC3/7 expression at selected time points during the 48hr window of drug exposure. The dose-response for each drug was used to select two concentrations of each drug that resulted in ~50% decrease in cell viability as a measure of cytotoxicity,

therefore 1 and 5µg/ml cisplatin, 0.2 and 0.4µg/ml doxorubicin and 0.01 and 0.5µg/ml vincristine concentrations were used. The GC-6spg cell line was cultured, chemotherapy drugs added and CellEVENT probe added as per Chapter 2 (see 2.6 and 2.8). The fluorescence of the cells were repeatedly measured at 6, 16, 24, 30, 40 and 48hrs after drug exposure with a microplate reader. Cell viability at the end of the culture period was determined through MTT assay to confirm the decrease in cell viability (see 2.7).

5.2.3.3: Experiment 5: Time-course of ROS production following chemotherapy treatment of GC-6spg cell line

The production of ROS was assessed with the use of the CellROX deep red reagent (Thermofisher, C10422) which is a fluorogenic probe that can be used in live cells and upon oxidation by ROS fluoresces deep red, but in a reduced state remains non-fluorescent. The fluorescent signal absorption/emission is ~644/665nm, which can be detected by use of microplate reader or fluorescent imaging. This probe was purchased diluted in DMSO with stock concentration of 2.5mM and was stored at -20^{o}C whilst protected from light. This probe was used under a class II laminar flow hood at all times. The CellROX probe was optimized for use on its own and also in combination with the CellEVENT probe.

5.2.3.3.1 Addition of CellROX or CellROX and CellEVENT Probes

The GC-6spp cell line culture was set up as previously described in Chapter 2 (2.6.6), with the drugs added on day two of culture. The concentrations of chemotherapy agents used include 1µg/ml cisplatin, 0.1µg/ml doxorubicin and 0.5µg/ml vincristine with clinical preparations of cisplatin and doxorubicin used as well as powder vincristine made up in PBS. Controls were exposed to equivalent volumes of PBS. At the selected time points during the 48hr culture period, the drug media was removed and replaced with fresh media containing either; the CellROX 5µM probe and DMSO, CellROX 5µM and CellEVENT 2µM probes or equivalent volumes of DMSO. The plates were returned to the incubator for 30mins to allow for uptake of the probes, after

which the probe media was removed and the wells washed three times with PBS. The DMSO wells were included to assess for autofluorescence of cells and blank wells were included to account for background fluorescence of plate. This culture was performed once and not repeated due to technical issues.

5.2.3.3.2 Measurement of Fluorescent Signal of CellROX probe

The fluorescent signal could not be measured with the use of a microplate reader, since it was not possible to access a reader with a filter with the correct absorption/emission spectrum. The fluorescent signal was therefore examined with use of Nikon A1R confocal microscope that had the correct filter cube fitted (Cy5). The confocal was set up for POC (perfusion open and closed) cell cultivation with caged incubator to allow for continued culture of cells at 32^{o}C, 5% CO_2. The images were initially taken at 10 or 20X magnification from the centre of a well. Additionally, Z-stacks of cells were performed at 20X. The intensity of the filter cubes were set at the start of each experiment and retained for all imaging. The brightness/contrast of representative images may be altered for illustrative/visualisation purposes.

5.2.3.3.3 Optimization

The use of the CellROX probe within our culture system was first optimized using the staurosporine drug as a 'positive' control, as used for optimization of the CellEVENT probe (see 2.8). The GC-6spg cell line was cultured as previously described in Chapter 2 (see 2.6) with cells either treated with 0.5µM staurosporine or equivalent volumes of DMSO. The CellROX or CellROX/EVENT probes were added (see above 5.2.5.2) at 2 or 4 hrs post-staurosporine exposure. In staurosporine treated wells, there was apparent signal from both the CellROX and CellROX+EVENT staurosporine treated cells compared to the controls at both the 2hr and 4hr time points (Fig 5.5). There was no obvious autofluorescence of cells with no probe in DMSO or staurosporine treated cells at either time point (Fig 5.5 i).

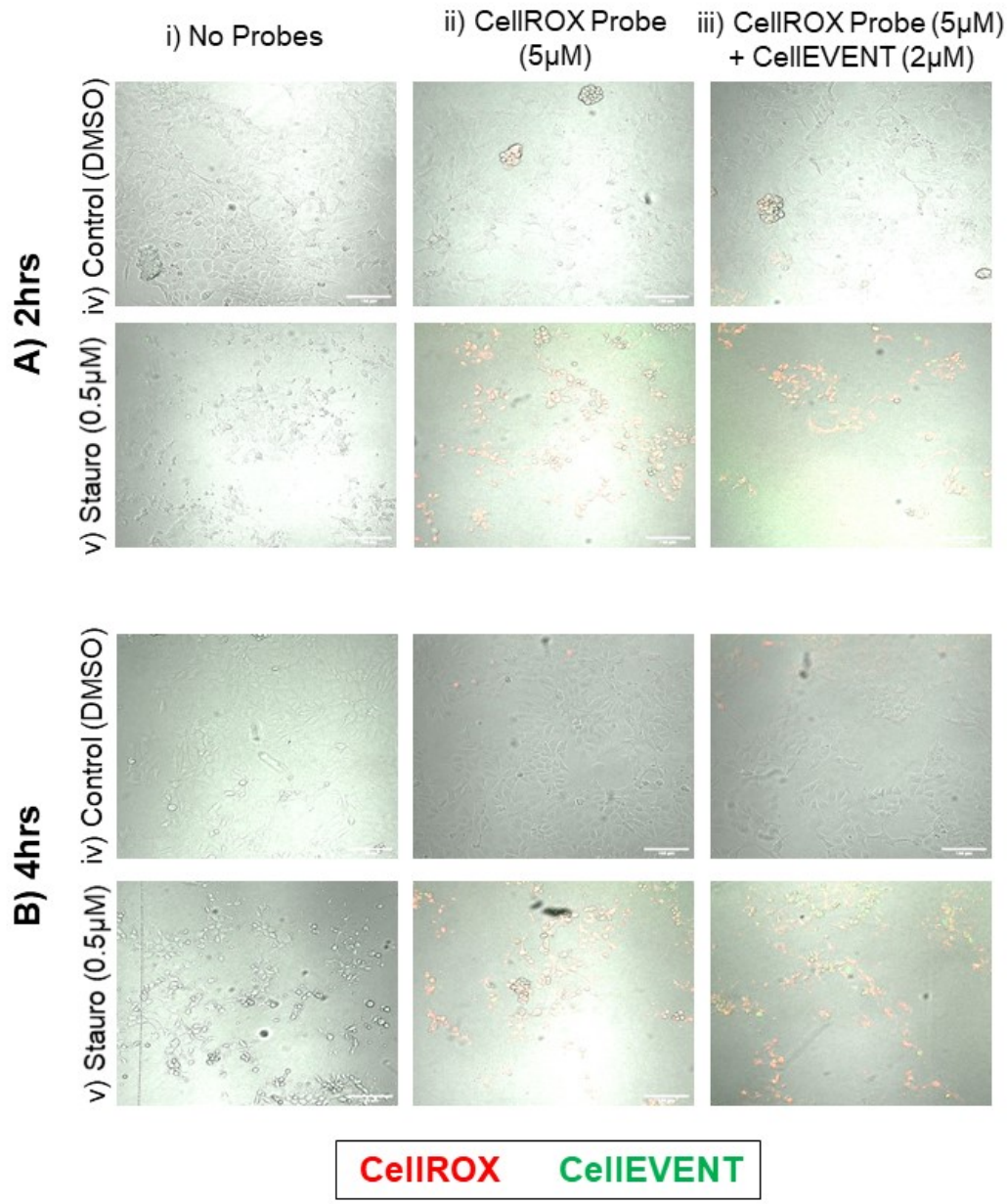


Figure 5.5: Optimization of CellROX and CellEVENT probes. Representative images of (iv) DMSO and (v) staurosporine treated GC-6spg cells with (i) no probes, (ii) CellROX probe or (iii) CellROX and CellEVENT probes. Fluorescence of probes (A) 2hrs or (B) 4hrs after exposure.

5.2.4 Statistics

Dose-response data for prepubertal testis tissue cultures were statistically analysed as described in Chapter 2.9 with one-way ANOVA where each concentration of drug compared to the controls. For the dose-response data for the GC-6spg cell line, data was compared with a one-sample t test or Wilcoxon test to the hypothetical control value of 100%. In addition to this analysis, the data was also fitted to a nonlinear regression curve with a dose-response sigmoidal model with a dose vs response (variable slope) equation. This analysis gave potential EC50 doses for each chemotherapy drug to be used in subsequent experiments (see Chapter 6.3).

5.3 Results

5.3.1 Dose-response for cisplatin or doxorubicin-induced loss of germ cells in the prepubertal testis

Analysis of oxidative stress following chemotherapy treatment of the prepubertal testis first requires concentrations at which gonadotoxic effects upon the germ cell population are apparent. Determination of a dose-response for cisplatin or doxorubicin treatment upon the prepubertal testis was therefore undertaken following 24hrs of treatment on day two of culture to select gonadotoxic concentrations for further experiments. The density of the germ cell population and proliferative activity of these cells were calculated per seminiferous tubule area at the end of a four-day culture period.

For cisplatin, a reduction in MVH-positive cells (green) is apparent at 'higher' concentrations of 0.5-10µg/ml cisplatin compared to control, as shown in representative images (Fig 5.6). The preliminary data indicates a loss of total germ cell density from 0.1 to 10µg/ml, with a specific reduction of the proliferating germ cell population (MVH+ (green), BrdU+ (red)) (Fig 5.7). Due to the low 'n' number ('n'=1-3), statistical analysis could not be undertaken; however, this data was generated for the purpose of indicating gonadotoxic concentrations for further experiments. Based on these initial results, gonadotoxic concentration of 1µg/ml cisplatin was chosen, which falls within the patient serum levels of this drug (0.05-4

µg/ml; Crom *et al.*, 1981; Dominici *et al.*, 1989; Peng *et al.*, 1997; Erdlenbruch *et al.*, 2001; Liston and Davis, 2017).

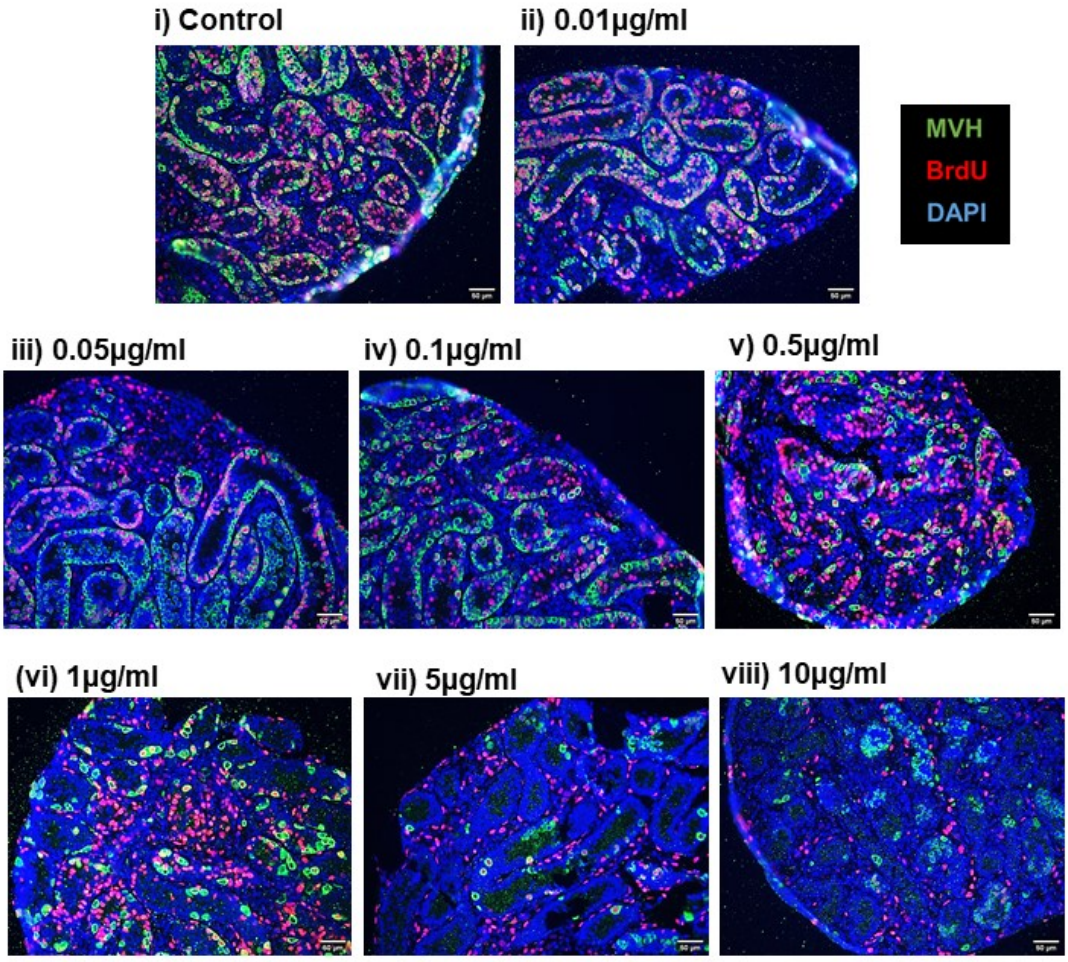


Figure 5.6: Representative images of dose-response prepubertal testis tissue at 0.01-10µg/ml cisplatin. (i) control and cisplatin treated tissues at concentrations from (ii) 0.01µg/ml to (viii) 10µg/ml. Images were taken at 20X for analysis and scale bar = 50µm. 'n'=1-3

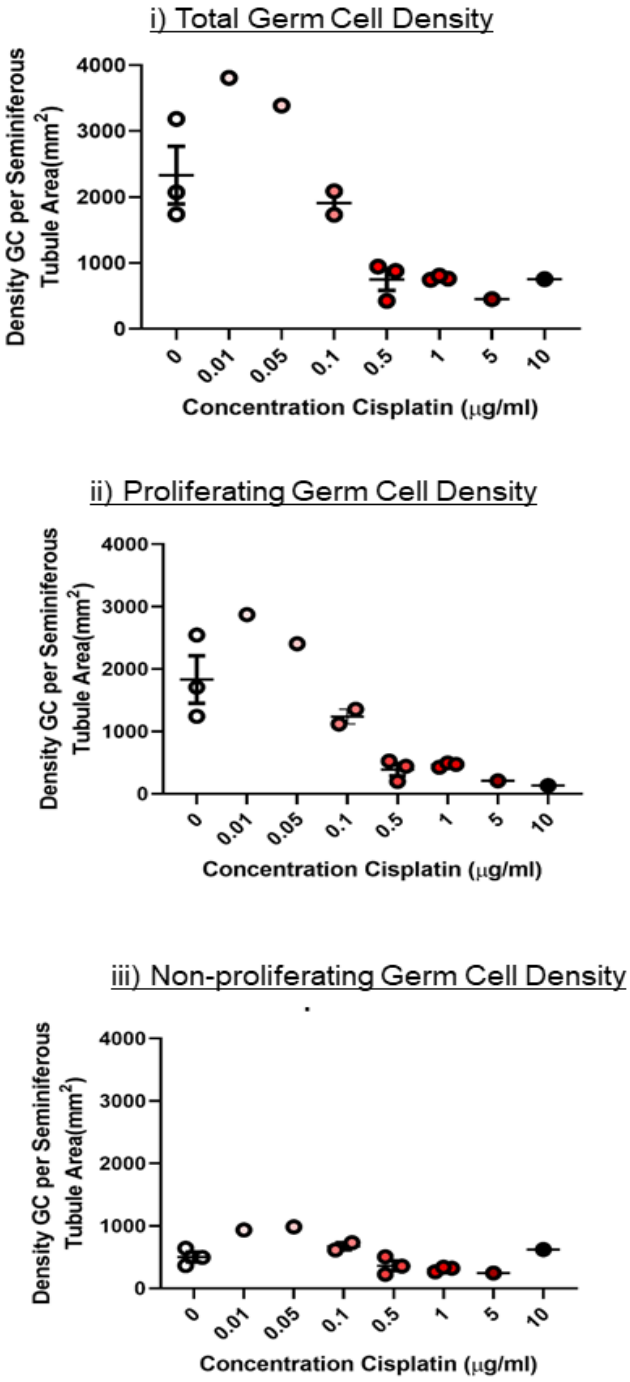


Figure 5.7: Analysis of dose-response prepubertal testis tissue at 0.01-10µg/ml cisplatin. (i) Germ cells (green MVH+) were manually counted per tubule area and classified as (ii) proliferating (MVH+, BrdU+) or (iii) non-proliferating (MVH+, BrdU-). The density of the total number and proliferating germ cells decreased with increasing concentrations of cisplatin. 'n'=1-3. Data shown as mean +/- SEM where relevant.

Doxorubicin-induced damage to the prepubertal testis was investigated utilizing both clinical and powdered preparations of the drug (0.005-0.5µg/ml) to determine the dose-response and rule out potential effects of excipients. A reduction in MVH-positive cells (green) was observed following powdered and clinical doxorubicin at 0.05 and 0.5µg/ml compared to the control, as shown in representative images (Fig 5.8). Analysis of density of germ cells and proliferative capacity of these cells for both powdered and clinical preparations produced similar trends with a reduction in total germ cell and proliferating germ cell densities from 0.05µg/ml (Fig 5.9 i, ii). Due to the low 'n' number for clinical data ('n'=2), statistical analysis was not performed. For powdered doxorubicin, there was a significant decrease compared to the control at 0.05µg/ml, with a 59% decrease in total germ cell density ($p=0.0019$), and a 73% decrease in the proliferating germ cell density ($p=0.0427$). Upon exposure to 0.5µg/ml powdered doxorubicin, the total germ cell density decreased by 77% ($p=0.0004$) and proliferating germ cell density by 88% ($p=0.0081$). There was no effect on the density of non-proliferating germ cells after powdered doxorubicin treatment ($p>0.05$). Based upon this preliminary data, a 'gonadotoxic' concentration of 0.2µg/ml was chosen for further experiments with doxorubicin, which falls within patient serum levels of this drug (0.02-3µg/ml; Greene *et al.*, 1983; Frost *et al.*, 2002; Hempel *et al.*, 2002; Krischke *et al.*, 2016; Liston and Davis, 2017) .

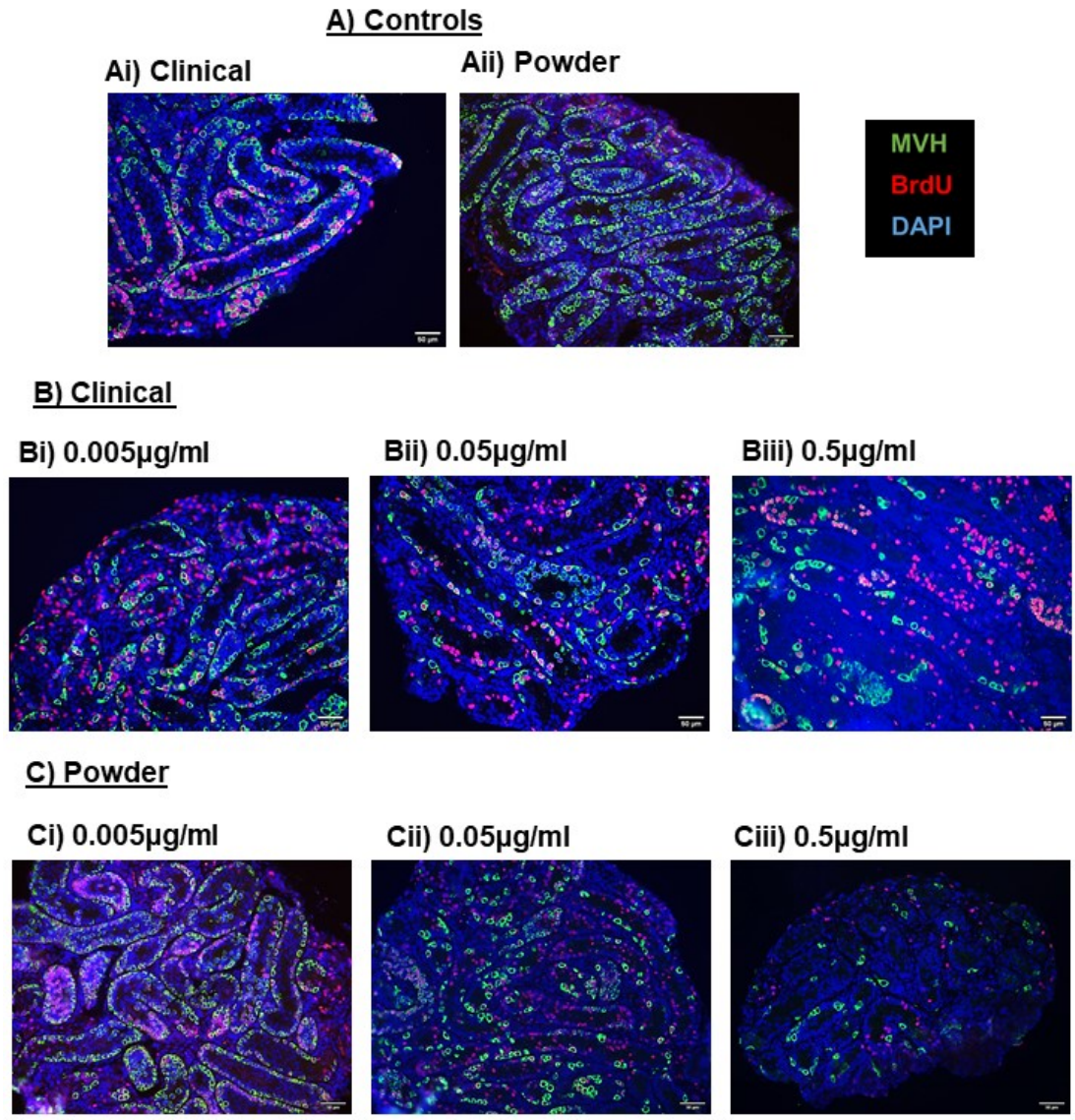


Figure 5.8: Representative images of dose-response of testis to 0.005-0.5µg/ml powder and clinical preparation of doxorubicin. Control tissue for (Ai) clinical and (Aii) powder preparation experiments. Representative images of doxorubicin treated tissues from (B) clinical and (C) powder preparations of doxorubicin from (B/Ci) 0.005µg/ml to (B/Ciii) 0.5µg/ml. Images were taken at 20X for analysis and scale bar = 50µm. 'n'=2 for experiments with clinical preparation and 'n'=4 for powder preparation of doxorubicin.

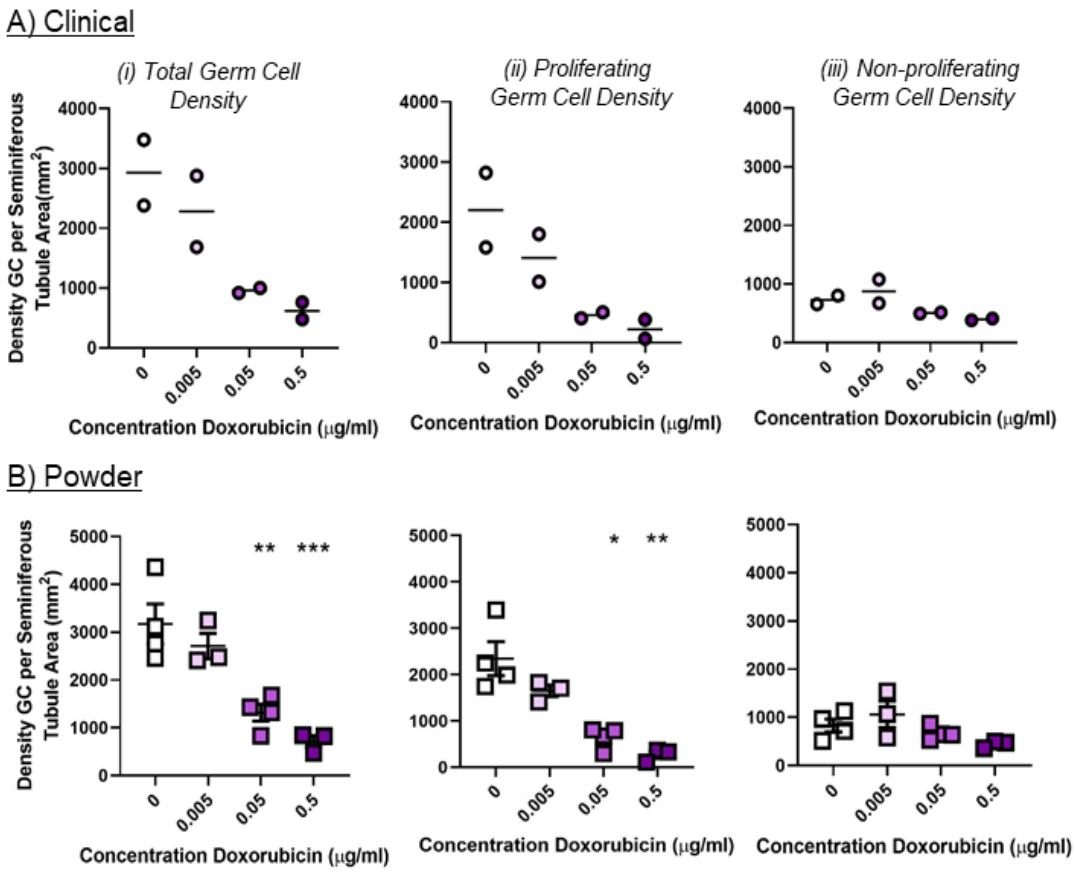


Figure 5.9: Analysis of dose-response of testis to 0.005-0.5µg/ml powder and clinical preparation of doxorubicin. (A/Bi) Germ cells (MVH+, green) were manually counted per tubule area and classified as (A/Bii) proliferating (MVH+ (green), BrdU+ (red) or (A/Biii) non-proliferating (MVH+ (green), BrdU-). The density of the total number and proliferating germ cells decreased with increasing concentrations of doxorubicin. 'n'=2 for experiments with clinical preparation and 'n'=4 for powder preparation of doxorubicin. See Appendix C Figure 2 for QQ-plots analysing normal distribution. Data analysed with one-way ANOVA followed by Dunnetts post-hoc test for total and non-proliferating germ cells. Data analysed Kruskal-Wallis tests followed by Dunn's multiple comparison test for proliferating germ cells. *p<0.05, ** p<0.01, ***p<0.001 comparing treatment groups to the control. Data are shown as mean +/- SEM.

5.3.2 Analysis of oxidative stress in prepubertal testis tissue following cisplatin, doxorubicin or vincristine treatment

To determine whether oxidative stress was induced following cisplatin, doxorubicin or vincristine treatment in the prepubertal testis, markers of oxidative stress were assessed during the 24hr exposure period and up to two-days after exposure of testis fragments to gonadotoxic concentrations of each drug. The gonadotoxic concentrations were chosen based on the dose-response studies and include 1µg/ml cisplatin, 0.2µg/ml doxorubicin and 0.5µg/ml vincristine. The time-course for oxidative stress was evaluated at 6, 16, 24, 30, 48 and 72hrs post-exposure to the gonadotoxic concentrations. Oxidative stress was measured by double immunofluorescent imaging of 8-OHdG (DNA/RNA damage, red) and MVH (germ cell, green), or MDA (lipid peroxidation, red) and MVH (germ cells, green) at each time point.

The impact on the germ cell density at gonadotoxic concentrations was confirmed by manually counting the number of MVH-positive cells (green) per section area on slides that were used for assessing 8-OHdG staining at each time point. Section area was utilized due to loss of testis architecture expected following vincristine exposure, as observed in Chapter 4 (see 4.3.1). An apparent decrease in the expression of MVH (green) was observed at the 72hr time when compared to the control for cisplatin- and doxorubicin-exposed tissues (Fig 5.10 Ai, Aii, Aiii). Preliminary data based on sample size ‘n’=3 further indicate a loss of germ cells at the 72hr time point compared to the controls for cisplatin- and doxorubicin-exposed tissues (Fig 5.11 Ai and Aii). The 72hr time corresponds to the end of the four-day culture period upon which the densities were assessed for the dose-response cultures. Based on these results, the gonadotoxic concentrations for cisplatin and doxorubicin are applicable for these time-course experiments. Following vincristine treatment, a similar observation was noted as in Chapter 4, whereby germ cells that originally were located along the basement membrane were found to have accumulated in the centre of seminiferous tubules (Fig 5.10 Aiv). This accumulation was observed as early as at the 16hr time

point (Fig 5.10 Aiv-Bii). Analysis of the densities of germ cells following vincristine treatment indicates loss of germ cells from the 16hr time point (Fig 5.11 Aiii). Based on these preliminary results, the concentration of vincristine chosen will be used for further experiments.

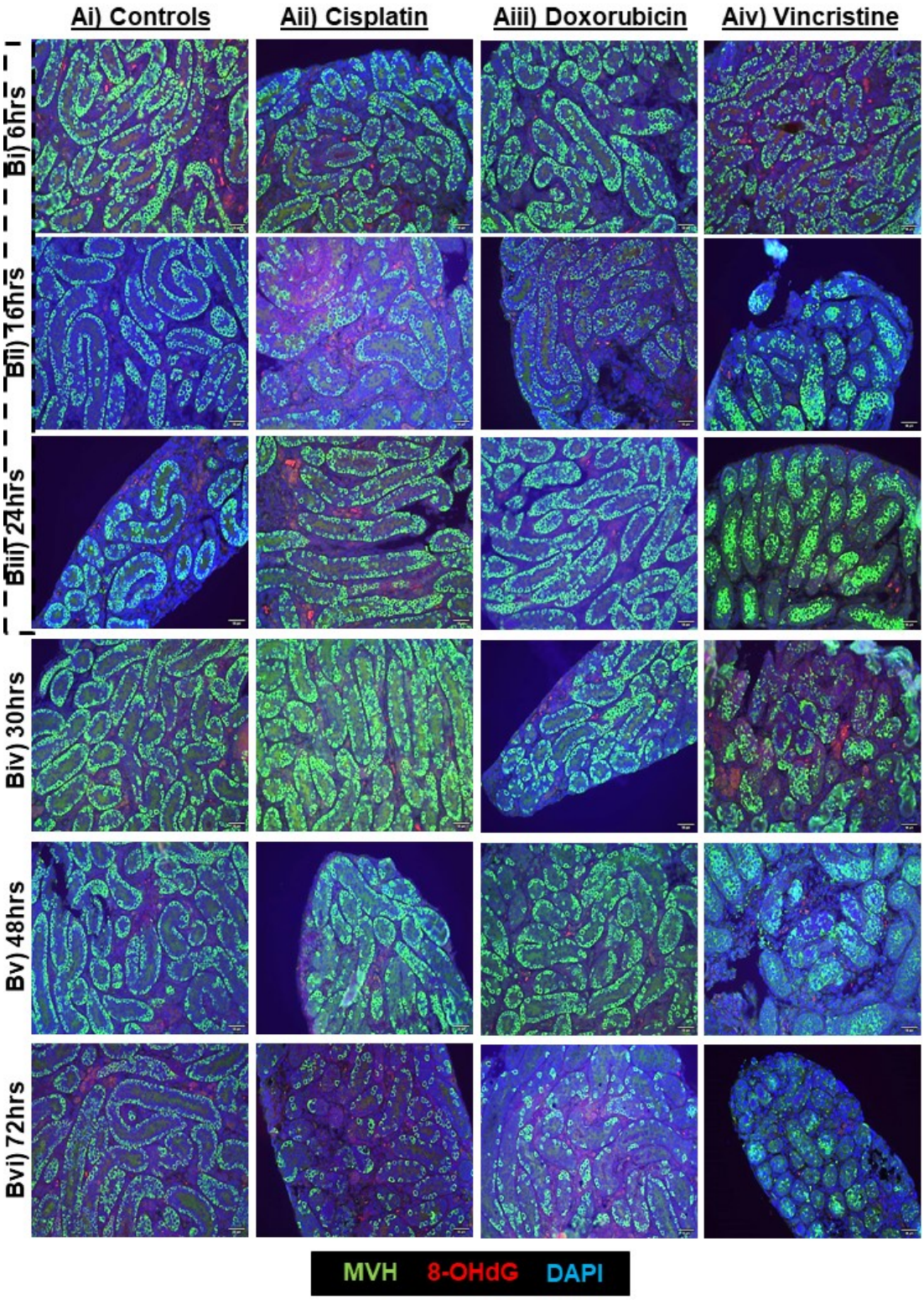


Figure 5.10: Time-course of 8-OHdG damage to the prepubertal testis tissue. Representative images of Ai) control, Aii) cisplatin, Aiii) doxorubicin or Aiv) vincristine treated tissues at Bi) 6hrs, Bii) 16hrs, Biii) 24hrs, Biv) 30hrs, Bv) 48hrs or Bvi) 72hrs after exposure to chemotherapy agents. Dashed box represents the times where tissues were cultured in drug media. 'n'=3.

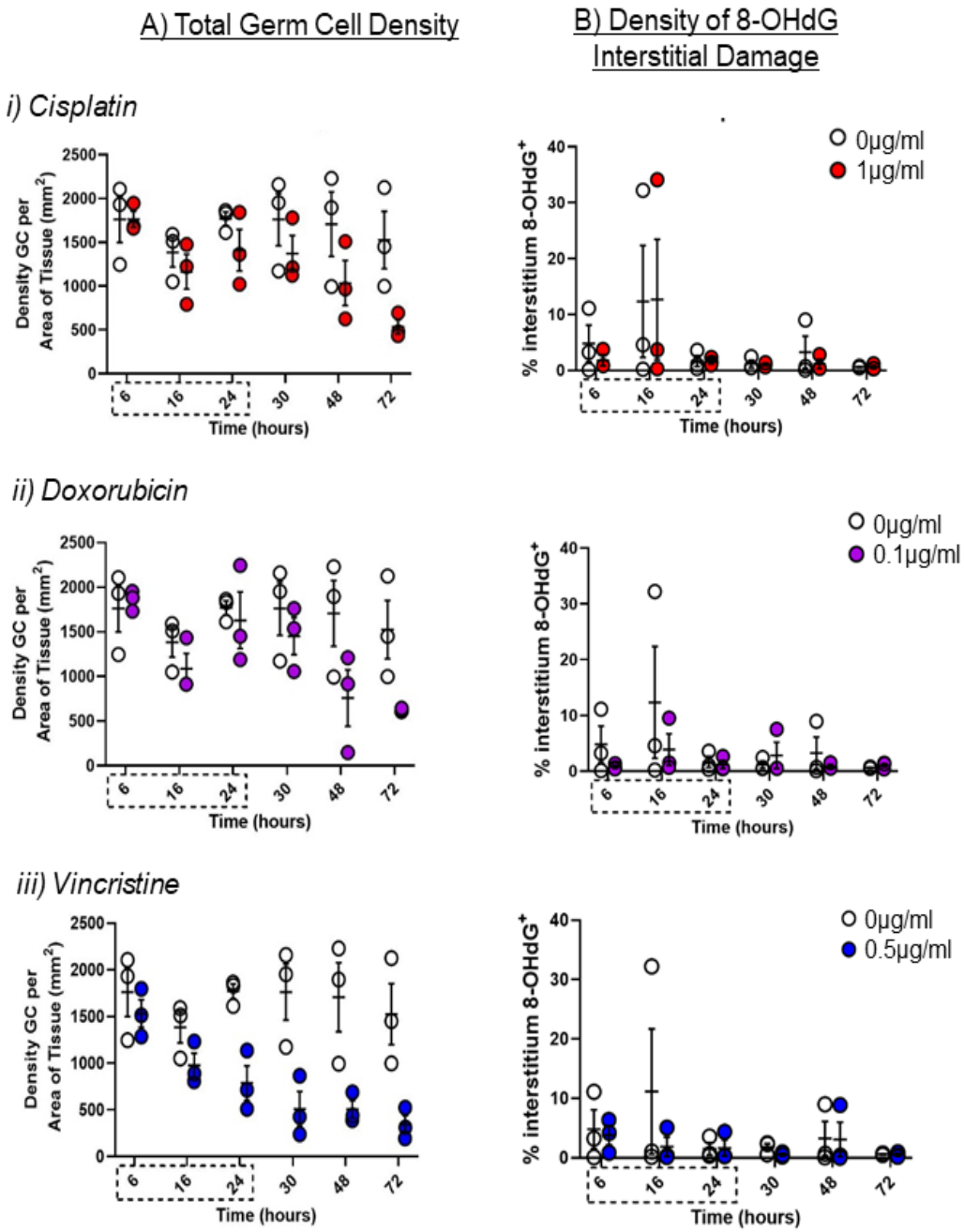


Figure 5.11: Analysis of germ cell density and 8-OHdG interstitial density. A) Densities of germ cells were manually counted per area of tissue following (i) cisplatin, (ii) doxorubicin or (iii) vincristine treatment. (B) Expression of 8-OHdG assessed per interstital area after chemotherapy exposure. 'n'=3. Dashed box represents the times where tissues were cultured within drug media.

Oxidative stress was assessed at the selected time points for DNA/RNA damage (8-OHdG) and lipid peroxidation (MDA). For the 8-OHdG experiment, observations from the assessor, who was blind to the experimental conditions, following the first run of immunofluorescence indicated a positive signal within the interstitium of the testis. Semi-automative analysis was therefore undertaken measuring the area of positive expression of 8-OHdG (red) to area of interstitium (Fig 5.10). The preliminary data generated, however, indicate that there is no trend in expression of 8-OHdG following cisplatin, doxorubicin or vincristine treatment (Fig 5.11 B). An increase in 8-OHdG was noted in an 'n' of 1 in both the control or cisplatin-exposed tissue fragments which may be an effect of culture. Assessment of lipid peroxidation was performed for an 'n' of 1, however, no obvious signal was observed in these testis tissue fragments, therefore further analysis was not undertaken (Fig 5.12). Biological controls of DMSO or B[a]P exposed tissues were included for each culture run, with B[a]P exposed tissues positive for both 8-OHdG and MDA compared to the DMSO treated tissues (see Fig 5.4B/Civ and v for examples).

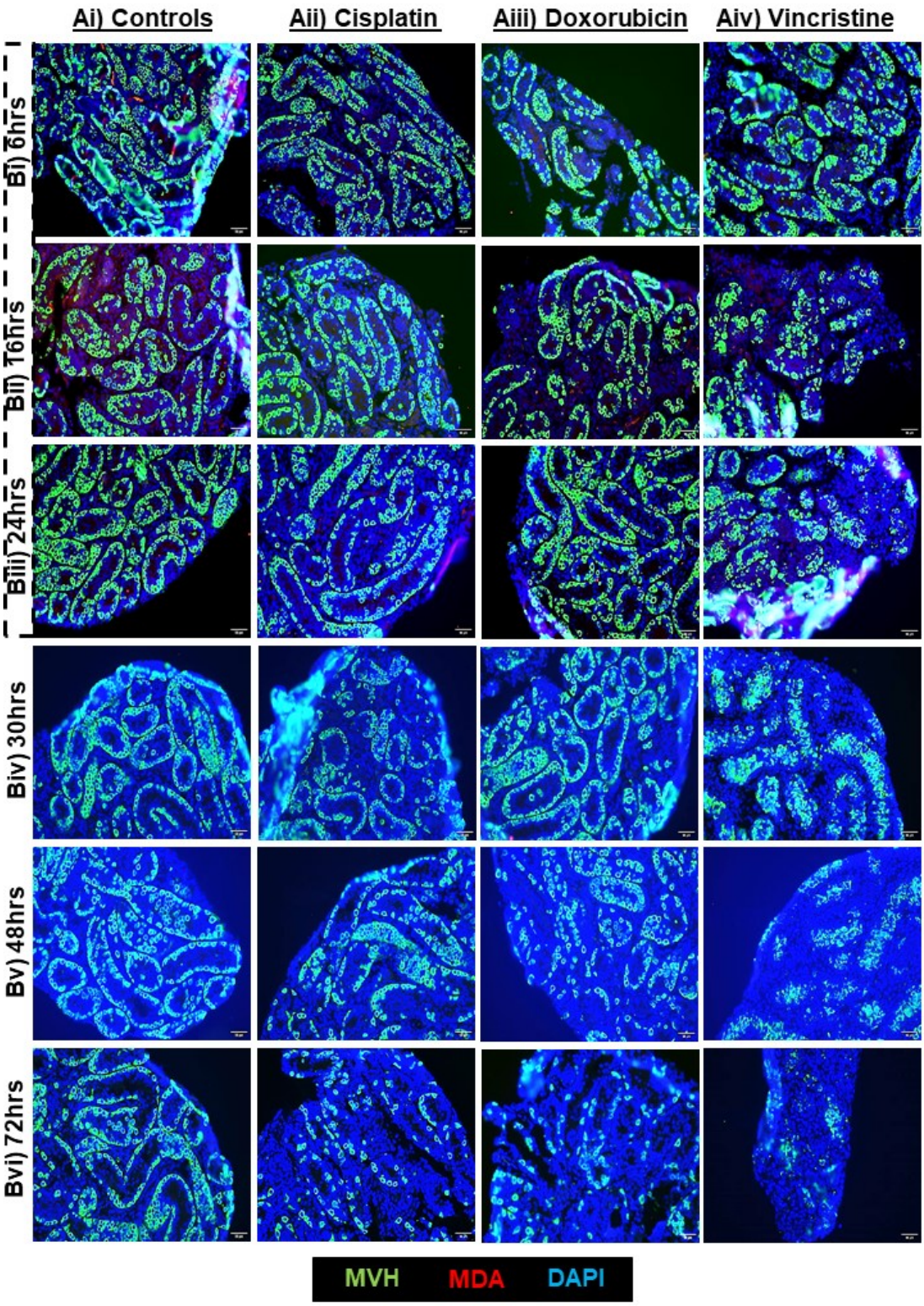


Figure 5.12: Time-course of MDA damage to prepubertal testis. Representative images of Ai) control, Aii) cisplatin, Aiii) doxorubicin or Aiv) vincristine treated tissues at Bi) 6hrs, Bii) 16hrs, Biii) 24hrs, Biv) 30hrs, Bv) 48hrs or Bvi) 72hrs after exposure to chemotherapy agents. 'n'=1. Dashed box represents the times where tissues were exposed to drug media.

5.3.3 Dose-response for cisplatin-, doxorubicin- or vincristine-induced cytotoxicity (reduced cell viability) to the GC-6spg cell line

A dose-response for cisplatin (0.01-10µg/ml), doxorubicin (0.05-5µg/ml) or vincristine (0.0001-10µg/ml) induced cytotoxicity was determined following treatment of the GC-6spg cell line for 48hrs. Dose-responses for each drug were generated to select EC50 doses for further experiments. Cytotoxicity was measured with an MTT assay, with the absorbance signal for control wells deemed 100% viable and absorbance of drug-exposed tissues compared to the control absorbance (shown as a percentage, compared to hypothetical 100% with one-sample t test). Following cisplatin treatment, significant decreases in cell viability were observed from 1µg/ml cisplatin, with 65 ± 7% cells viable after treatment ($p= 0.002$) (Fig 5.13 Ai). The cell viability of doxorubicin-exposed cells was reduced from 0.05µg/ml doxorubicin, with 65 ± 2% cell viability ($p<0.0001$) (Fig 5.13 Aii). Finally, vincristine treatment had a significant effect on cell viability from 0.5µg/ml, with 65 ± 14% cells remaining viable ($p=0.0148$) (Fig 5.13 Aiii). The dose-response curves for both cisplatin and doxorubicin follow a similar trend with cell viability reducing to around 0 to 5% at the highest concentrations tested for cisplatin (10µg/ml, not shown in Fig 5.13 due to 'n'=1) or doxorubicin (0.4µg/ml, Fig 5.13 Aii). However, the dose-response for vincristine followed a different pattern where cell viabilities at the highest concentrations tested remained around 40% (Fig 5.13 Aiii).

Further experiments with the GC-6spg cell line required concentrations at which cell viability is reduced by ~50% (EC50 dose) to determine the role of ROS in cisplatin, doxorubicin or vincristine-induced cytotoxicity. The above data was processed in GraphPad Prism to fit a curve with non-linear regression with a dose vs response (variable slope) equation. EC50 doses generated for each drug are as follows; cisplatin (EC50 = 7µg/ml, Fig 5.13 Bi), doxorubicin (EC50 = 0.2µg/ml, Fig 5.13 Bii) or vincristine (EC50 = 0.001µg/ml, Fig 5.13 Biii). The confidence interval, however, for these EC50 doses are very wide and could not be computed, due to the low 'n' number for some concentrations included. Therefore, the EC50 doses for further experiments were

chosen taking into account the analysis by one-sample t test and the dose-response curves with EC50 doses. For each drug, two concentrations were chosen due to the ambiguity with some of the data points, therefore 1 and 5µg/ml cisplatin, 0.2 and 0.4µg/ml doxorubicin as well as 0.01 and 0.5µg/ml vincristine were used for further experiments.

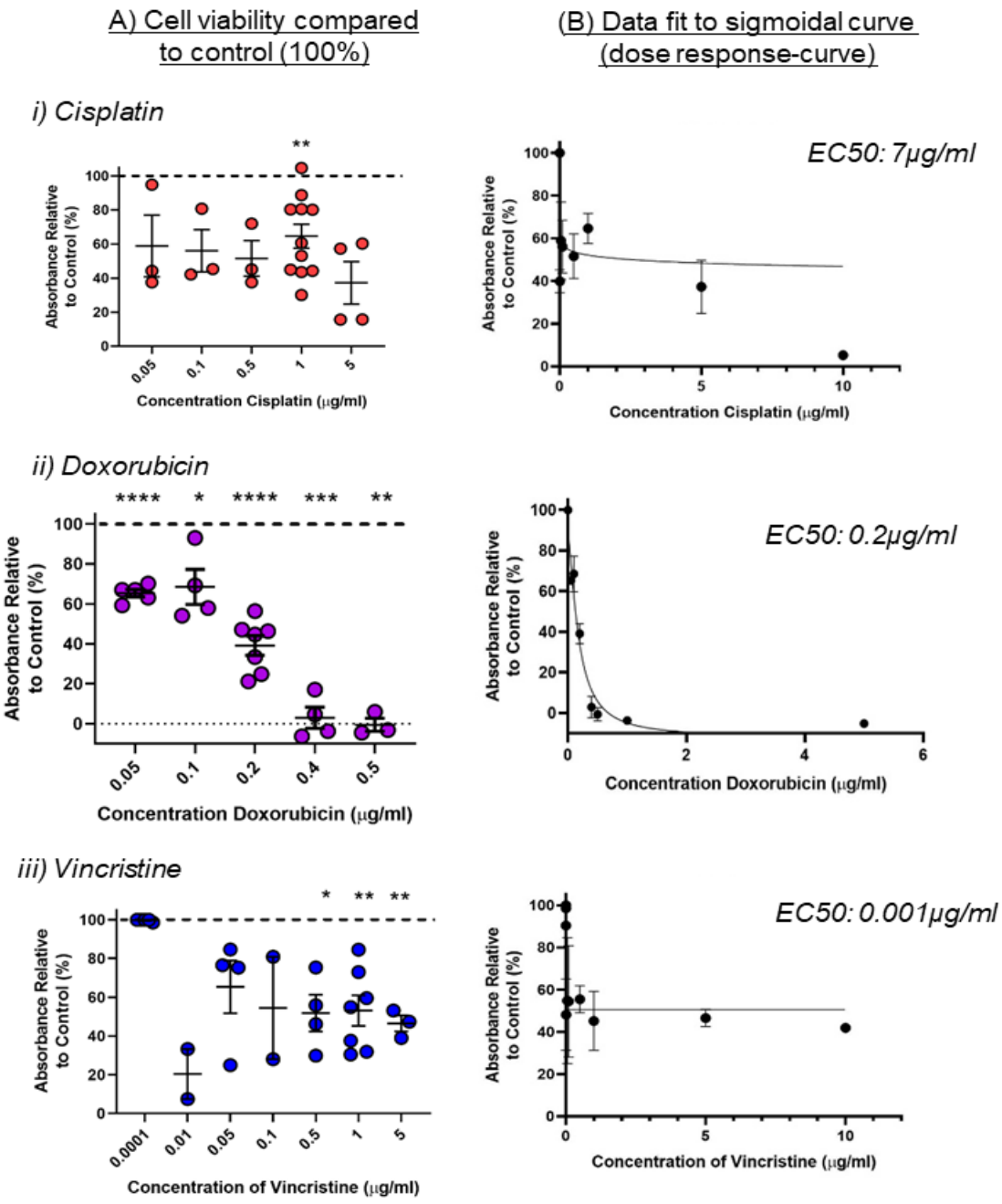


Figure 5.13: Dose-response for cisplatin, vincristine and doxorubicin-induced damage to the GC-6spg cell line. (A) MTT assay used to calculate cell viability per the control set to 100% viability (dashed line at 100%) following (i) cisplatin, (ii) doxorubicin or (ii) vincristine treatment of the GC-6spg cell line. See Appendix C Figure 3 for QQ-plots analysing normal distribution. Data compared to hypothetical mean of 100% with one-sample t-test for doxorubicin and vincristine, whereas a one-sample Wilcoxon test was used for cisplatin data. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Individual data points shown +/- SEM. (B) Data was analysed with sigmoidal dose-response curve on Graphpad, which generated a EC50 dose. Data shown as mean +/- SEM. 'n'=1-11, (culture runs with data points representing average of technical replicates).

5.3.4 Time-dependent analysis of apoptosis following cisplatin, doxorubicin or vincristine exposure in GC-6spg cell line

The time-course of apoptosis following cisplatin (1 and 5µg/ml), doxorubicin (0.2 and 0.4µg/ml) or vincristine (0.01 and 0.5µg/ml) treatment of the GC-6spg cell was determined at selected time points at 6, 16, 24, 30, 40 and 48hrs post-exposure. Apoptosis was assessed through use of CellEVENT probe that fluoresces upon activation of CC3 and 7 with use of microplate reader. This data will determine the timing of apoptosis and indicate whether the loss of cell viability described above is attributable to chemotherapy-induced apoptosis. This data is preliminary as results were produced from three culture runs and therefore statistical analysis was not performed. An MTT assay was performed after the 48hr drug exposure to confirm a reduction in cell viability. Trends in the data were therefore determined by comparing the overlap of the SEM for data points. For cisplatin-exposed tissues, activation of apoptosis was observed from 40hrs post-exposure with 5µg/ml compared to the control (Fig 5.14 A 40hrs). Doxorubicin-induced increase in activation of apoptosis followed a different pattern, as the signal appeared to increase from as early as the 24hr time point for 0.4µg/ml (Fig 5.14 A, 24hrs). The 0.2µg/ml doxorubicin apoptosis activation increased as well from the 24hr time point but to a lesser extent and produced a variable signal between the three cultures (Fig 5.14 A). Apoptosis following vincristine treatment was observed at the 48hr time point for both 0.01 and 0.5µg/ml; however, the increase signal was not as obvious as other chemotherapeutic agents (Fig 5.14 A, 48hr). The MTT assay performed at the 48hr time point indicate that 5µg/ml cisplatin, 0.2µg/ml doxorubicin and 0.5µg/ml vincristine are representative of EC50 doses and would enable comparison between the different chemotherapy drugs (Fig 5.14 B).

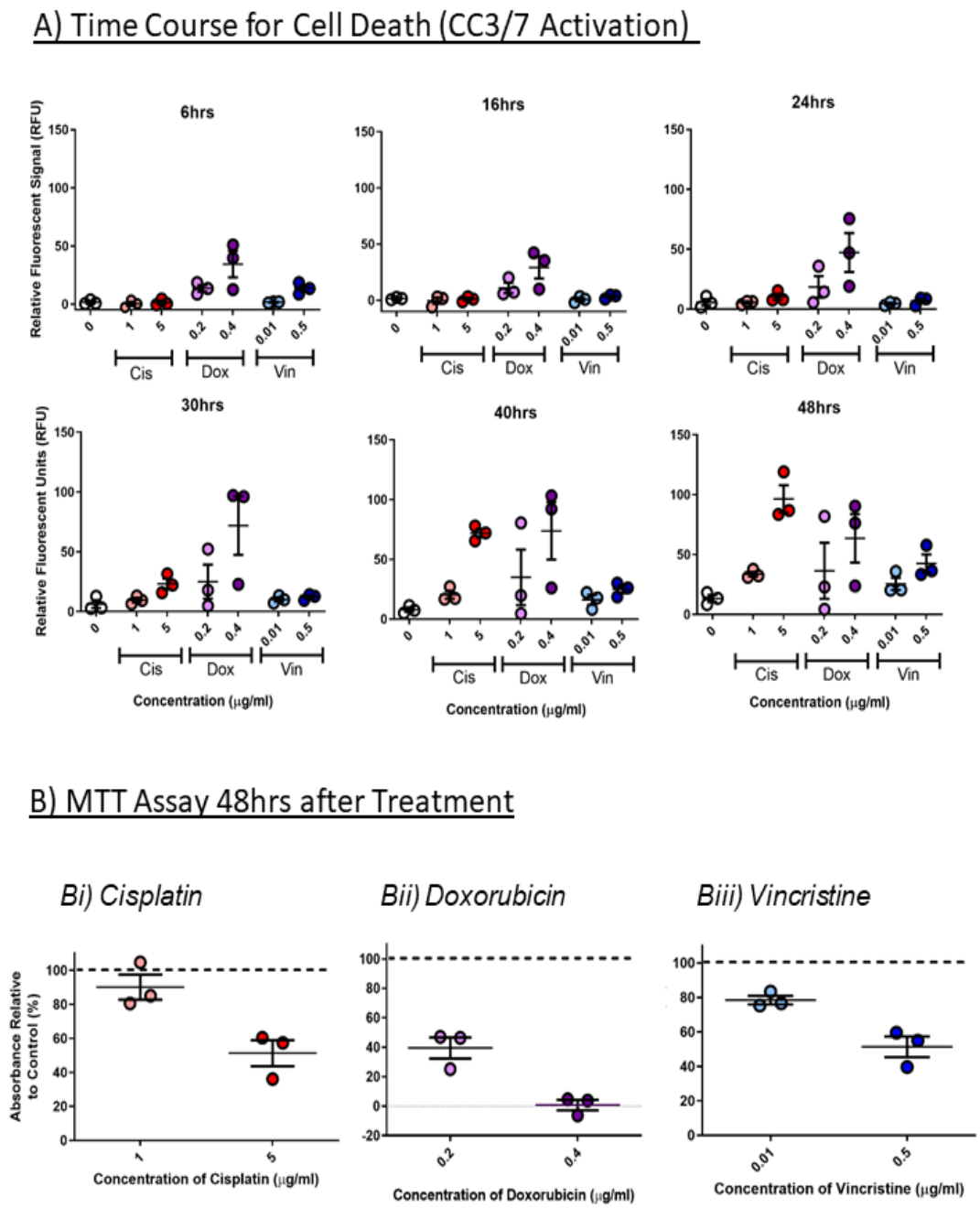


Figure 5.14: Time-course of CC3/7 expression of GC-6spg cell following cisplatin, doxorubicin or vincristine-induced damage. A) CellEVENT probe (fluoresces upon activation of CC3/7) was added to the start of culture and signal analysed at 6, 16, 24, 30, 40 and 48hrs after exposure to cisplatin (cis, red), doxorubicin (dox, purple) or vincristine (vin, blue). (B) MTT assay used to assess cell viability at 48hr time points following (i) cisplatin, (ii) doxorubicin or (iii) vincristine treatment. 'n'=3 (culture runs with data points representing average of technical replicates). Due to low 'n' number, differences between data points were compared by assessing overlap of SEM.

5.3.5 Steps towards analysis of ROS production following cisplatin, doxorubicin or vincristine exposure in GC-6spg cell line

Assessment of production of ROS following cisplatin (1µg/ml), doxorubicin (0.1µg/ml) or vincristine (0.5µg/ml) exposure was determined with CellROX probe that fluoresces deep red upon oxidation. These concentrations differed to those in experiments above (5.3.4) due to early assessment of the dose-response data that indicated these doses represented the EC50, however upon further experiments selected EC50 doses were changed for CellEVENT experiments (above 5.3.4). These experiments aimed to establish whether the chemotherapy drugs induce ROS, and whether this occurred before or after the activation of apoptosis, therefore the CellEVENT probe was also added to the culture. The level of ROS production and apoptosis activation was assessed at 6, 24 and 48hrs post chemotherapy drug exposure in the GC-6spg cell line. Initially, images for analysis were taken at a single plane where positive CellROX signal was observed to an extent in most images taken, including control and chemotherapy treated tissues (Fig 5.15). The most abundant CellROX signal was detected in the vincristine treated tissues; however, as these images are representative of a single culture, no definitive conclusions can be drawn. CellEVENT signal was apparent in the vincristine treated tissue at 48hr time point. Based upon these observations from the initial culture, issues arose as to how to analyse this signal and as to whether analysis from a single image from one focal plane chosen by the assessor was appropriate. An additional culture was therefore undertaken where only the CellROX probe was analysed. Images from this culture were taken ~27hrs after chemotherapy exposure with a Z-stack where individual images were taken across a defined focal range and combined to produce the final image. A signal was observed in the control, doxorubicin and vincristine-exposed tissues (Fig 5.16). An appropriate way to measure this signal, however, could not be determined and time constraints did not enable further optimization.

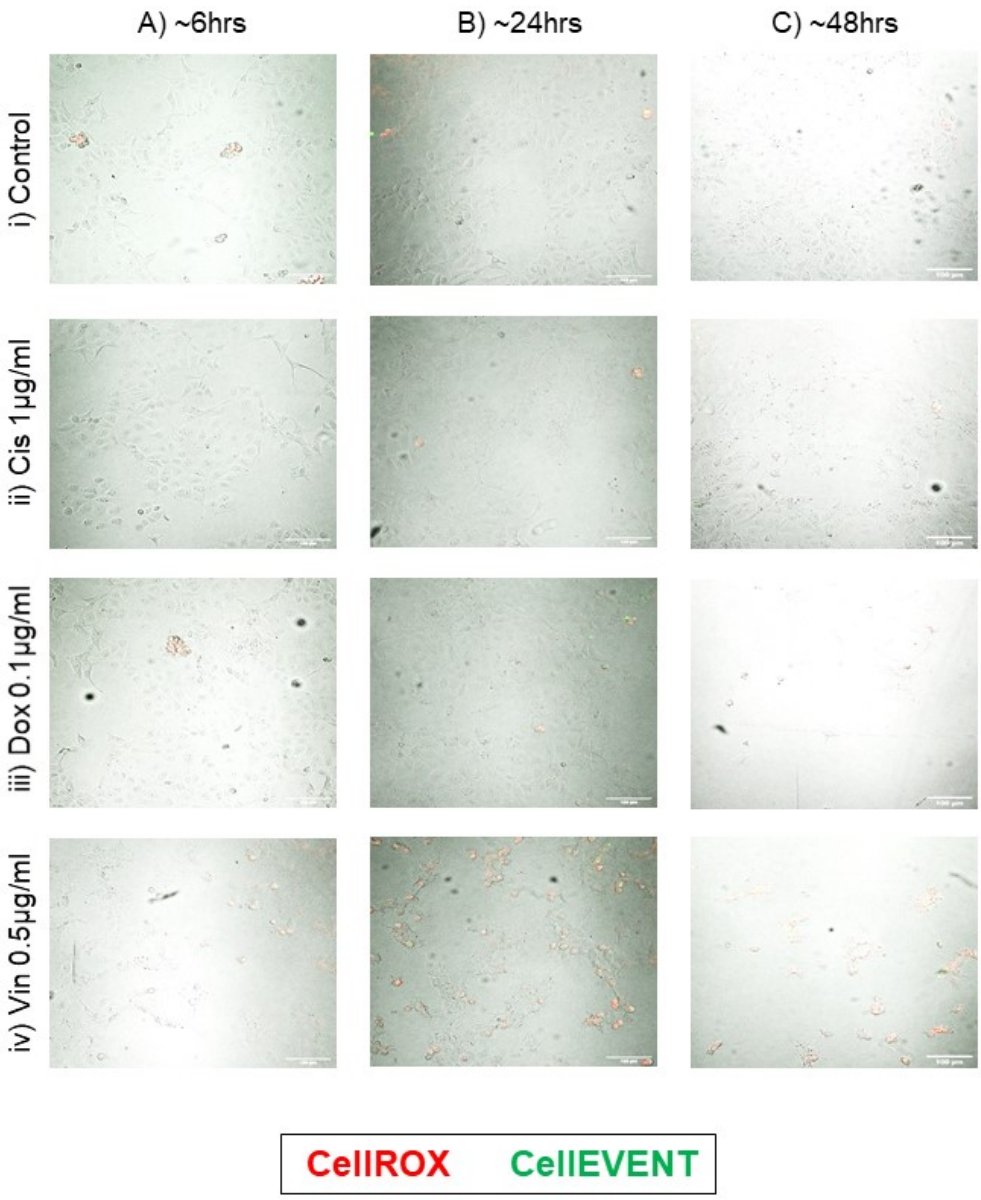


Figure 5.15: Time-course of ROS (CellROX) and CC3/7 (CellEVENT) expression in GC-6spg cell line. Representative images of GC-6spg cell line following (A) 6hrs, (B) 24hrs or (C) 48hrs exposure to either (i) control, (ii) cisplatin, (iii) doxorubicin or (iv) vincristine. 'n'=1

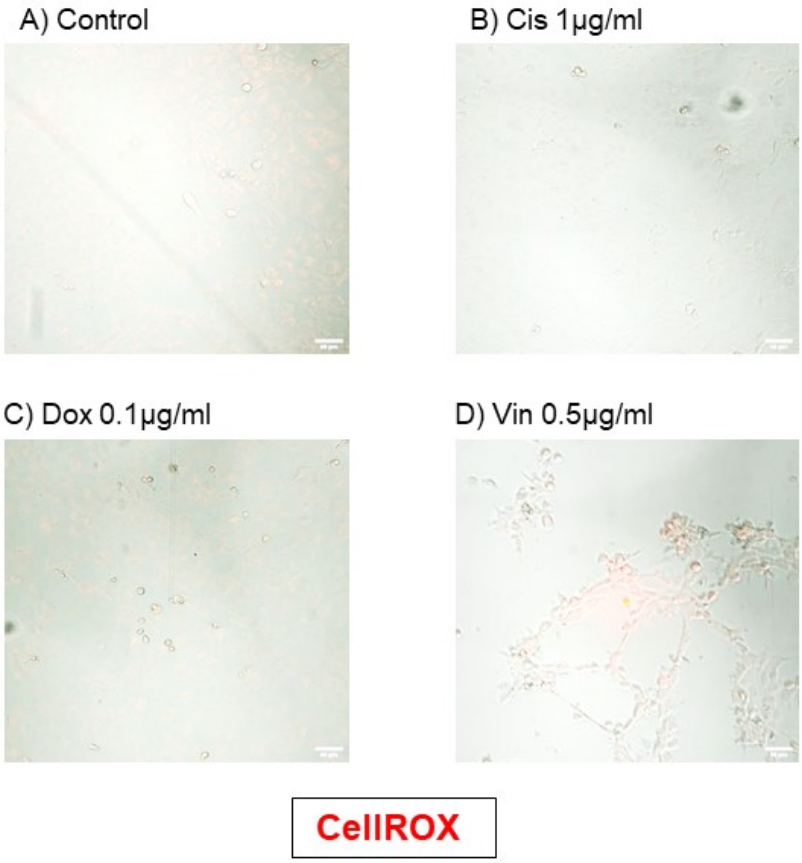


Figure 5.16: Z-stack of ROS expression following cisplatin, doxorubicin or vincristine treatment in the GC-6spg cell line. Representative images of (A) control, (B) cisplatin, (C) doxorubicin or (D) vincristine treated GC-6spg cells with CellROX probe added to culture. 'n'=1

5.4 Discussion

5.4.1 Overview of Results

Understanding the underlying molecular and mechanistic pathways of chemotherapy-induced damage to specific tissues is an essential step towards developing chemo-protectants to prevent toxic side effects of treatments, including gonadotoxicity. Within this chapter, the role of ROS and oxidative stress in cisplatin-, doxorubicin- or vincristine-induced damage to the prepubertal testis was assessed with use of two culture models. Oxidative stress was analysed in cultured fragments of mouse prepubertal testicular tissues that were exposed to chemotherapeutic agents for 24hrs. Markers of oxidative stress analysed include 8-OHdG (DNA/RNA

damage) and MDA (lipid peroxidation); production was measured at specific time points during the course of the four-day culture by immunohistochemistry. The preliminary data, however, indicates no apparent increase in oxidative stress (upon analysis of chosen oxidative stress markers) following treatment with gonadotoxic concentrations of each drug. Oxidative stress is a result of ROS production; however, these reactive species are difficult to measure due to their transient nature. Use of fluorescent probes in cell culture experiments to asses ROS have been successful, therefore further cultures were undertaken with a rat, SSC representative, cell line (GC-6spg). Fluorescent probes were used to detect production of ROS and activation of apoptosis in a time-dependent manner to determine the role of ROS in stimulating apoptosis in cisplatin-, doxorubicin- or vincristine-induced cytotoxicity. The preliminary data generated shows a trend towards activation of apoptosis, through increased expression of CC3/7; however, whether ROS stimulates apoptosis or is produced as a by-product could not be determined within this study.

5.4.2 Dose- and time-dependent effects of cisplatin, doxorubicin or vincristine treatment on the germ cell population of prepubertal testis

Assessment of oxidative stress was undertaken at gonadotoxic concentrations that were determined following an initial dose-response study of cisplatin (0.01-10µg/ml) or doxorubicin (0.005-0.5µg/ml) upon the prepubertal mouse testis (see Chapter 4 for vincristine dose-response, 4.3.1). The concentrations tested were within paediatric patient serum levels of cisplatin (0.05 - 4µg/ml; Crom *et al.*, 1981; Dominici *et al.*, 1989; Peng *et al.*, 1997; Erdlenbruch *et al.*, 2001; Liston and Davis, 2017) and doxorubicin (0.02-3µg/ml; Greene *et al.*, 1983; Frost *et al.*, 2002; Hempel *et al.*, 2002; Krischke *et al.*, 2016; Liston and Davis, 2017). Our group has previously published dose-responses for cisplatin and doxorubicin with the pnd five prepubertal mouse testis culture system, where similar reductions in germ cell (MVH-positive cells) densities were observed at the concentrations tested here (Smart *et al.*, 2018). In addition, the Smart *et al.* (2018) study confirmed a specific loss of the SSC population with no effect upon the densities of somatic cells, including Sertoli and Leydig cells.

The findings, however, did not rule out the possibility of late effects and/or functional deficits in the somatic population. For example, functional aberrations following treatment, including reduced production of transferrin or androgen binding protein (ABP), has been observed following cisplatin or doxorubicin exposure, *in vivo,* in prepubertal animal model studies (Favareto *et al.*, 2011; Brilhante *et al.*, 2012). Dose-response cultures were initially repeated to confirm the germ cell findings with use of clinical preparations of cisplatin or doxorubicin, and when culturing larger ($1mm^3$) testis fragments at lower temperature (34^oC). The 'n' number of these experiments were therefore low. Based upon on the dose-response data generated within this thesis, gonadotoxic concentrations of cisplatin (1µg/ml), doxorubicin (0.2µg/ml) and vincristine (0.5µg/ml), which are within serum levels, were chosen to further investigate in a time-course study to assess oxidative stress.

The time-course study collected tissue at 6, 16, 24, 30, 48 and 72hrs post-exposure to the chemotherapy agents, encompassing the four-day culture period. Analysis of the germ cell densities were performed to confirm the gonadotoxic effects of the concentrations chosen from the dose-response culture. The preliminary findings show an apparent reduction in MVH-positive germ cells by the 72hr time point following cisplatin or doxorubicin treatment. Exact timings; however, of germ cell loss would require a larger 'n' number. With a higher 'n' number, analysis undertaken would include a two-way ANOVA, taking into account the post-exposure time and concentrations of drugs. Smart *et al.* (2018) performed a similar time-course study with lower doses than those used within in our study, including 0.1µg/ml cisplatin and 0.05µg/ml doxorubicin, with tissue collected in eight-hr intervals up to 40hrs after chemotherapy drug exposure. Reductions in germ cell densities were apparent from 32hrs post-exposure to cisplatin and after 40hrs doxorubicin exposure. Comparisons, however, between these two studies need to take into account the different methods of analysis of MVH expression (manual counting per area section here versus semi-automative analysis per tubule area), doses, incubator temperature and size of tissue fragments. Analysis of apoptosis and DNA damage were also

performed showing increased expression of CC3 eight hrs before loss of germ cells following cisplatin treatment; with increased DNA damage (assessed through γH2AX expression) 16hrs after cisplatin or doxorubicin exposure (Smart *et al.*, 2018). There was no apparent increase in CC3 expression upon doxorubicin treatment within the study before germ cell loss, further analysis of our time-course with markers of apoptosis would therefore be of interest to determine whether such enhanced CC3 expression is apparent at the time points assessed here. To date, there has been no studies to this author's knowledge that have assessed other molecular targets of cisplatin- or doxorubicin-induced toxicity to the prepubertal testis, highlighting the need for studies such as those performed within this thesis for oxidative stress as a potential target.

Vincristine was initially included in experiments investigating the role of oxidative stress as a control drug, since this agent was considered to induce lows level of oxidative stress (reviewed in Conklin, 2004). The results of vincristine exposure in the prepubertal mouse testis have been described in Chapter 4. Germ cells were found to have accumulated in the centre of seminiferous tubules at the end of the four-day culture. This time-course study, therefore, enabled assessment of the timing and potential events leading to this accumulation. The movement of germ cells into the centre of the seminiferous tubules was apparent as early as the 16hr time point from 'n'=3. Further analysis of this time-course with markers of cell death (e.g. CC3) or cell cycle (e.g. FUCCI, fluorescent ubiquitination-based cell cycle indicator reviewed in Zielke and Edgar, (2015)) would determine whether these cells are undergoing delayed cell death, have exited the cell cycle or have lost contact with the basement membrane/Sertoli cells through other mechanisms leading to these observations (see Chapter 4, 4.4.2 for more details). Confirmation of germ cell loss and/or accumulation verifies the gonadotoxic findings from our dose-response analysis, indicating that the concentrations used within the time-course study are appropriate for assessing the events leading to gonadotoxicity following cisplatin, doxorubicin or vincristine treatment.

5.4.3 Oxidative stress in mouse prepubertal testis upon cisplatin, doxorubicin or vincristine treatment

Markers of lipid peroxidation and DNA/RNA damage were used to assess oxidative stress at 6, 16, 24, 30, 48 or 72hrs after drug exposure. The preliminary results, however, show no increase in formation of MDA (lipid peroxidation) or 8-OHdG (DNA/RNA damage) following cisplatin, doxorubicin or vincristine treatment. This would therefore suggest that oxidative stress is not involved in the underlying mechanism of action of cisplatin or doxorubicin-induced gonadotoxicity, since loss of the germ cells were apparent by the final time point assessed at 72hrs. This data is preliminary with additional cultures required to confirm these initial findings, as well as analysis with alternative markers which are available to study oxidative stress through immunohistochemistry (reviewed in Liou and Storz, 2015).

In prepubertal animals, to date, oxidative stress following cisplatin or doxorubicin exposure has been explored in studies using pnd 30 prepubertal rats, with increased formation of MDA and reduced antioxidants levels following treatment noted (Cabral *et al.*, 2018; Abdelaziz *et al.*, 2019; Aboul-Naga *et al.*, 2020). Cisplatin- or doxorubicin-induced oxidative stress has been assessed more thoroughly following treatment of the adult testis of animal models *in vivo.* Key word searches in NCBI database in July 2020 for 'chemotherapy drug' AND oxidative stress AND testis generates a number of papers related to this topic for cisplatin (67 papers) or doxorubicin (47 papers), with no such papers for vincristine. The majority of studies show increased lipid peroxidation and DNA/RNA damage, as well as decreased activity of antioxidants following cisplatin or doxorubicin treatment whilst investigating the protective effects of a number of antioxidants (Ateşşahin *et al.*, 2006a, b; Prahalathan *et al.*, 2006; Türk *et al.*, 2008; Das *et al.*, 2012; Köroğlu *et al.*, 2019; Eren *et al.*, 2020). Measurements of oxidative stress and antioxidants performed by the vast majority of these studies, and those in prepubertal animals, used homogenized whole testes and determined activity or presence of oxidative stress or antioxidants through

biochemical assays, ELISAs or Western blots in a set volume/weight of homogenate. This approach has a major drawback as the specific cell type within the testis producing the resultant oxidative stress cannot be determined. Within this study, therefore, double immunofluorescence was utilized to determine in which testicular cell the oxidative stress was induced, focusing upon the germ cell population as there is a clear effect on density following treatment. The small size of our testis fragment, however, may not have generated sufficient production of MDA or 8-OHdG that could be visualised with immunofluorescence. Analysis of 8-OHdG through immunohistochemistry was performed by Eren *et al.* (2020) in adult testes from cisplatin-exposed rats; a positive DAB signal within the germ cells and Leydig cells were observed seven days after treatment, representing oxidative stress. Our initial observations of experimental images for analysis, with assessor blind to experimental conditions, showed a positive signal within the interstitial compartment of the prepubertal testis that may be indicative of Leydig cell damage, as shown in the adult testis in Eren *et al.* (2020). The preliminary findings, however, were not associated with increased interstitial signal following chemotherapy treatment. In regards to the germ cell population, from the representative images in Eren *et al.*, (2020), the 8-OHdG positive signal is not observed in the germ cells adjacent to the basement membrane, where spermatogonia are located. Instead, 8-OHdG expression is limited to those germ cells towards the centre of the seminiferous tubules, the meiotic germ cells or spermatozoa. This is not discussed within the Eren *et al.* (2020) study, and therefore may not have been observed in different animals. Analysis of testis tissue from *in vivo* experiments following chemotherapy treatment of prepubertal animals may be a more appropriate methodology if expression levels are indeed an issue. Alternatively, combining multiple fragments from cultures exposed to the same conditions could produce enough tissue to generate sufficient homogenate to test through biochemical, ELISA or Western blot techniques

Dose- and time-dependent factors could also explain the lack of any observable oxidative stress following chemotherapy treatment in the cultured prepubertal testis

fragments. The role of oxidative stress in doxorubicin-induced cardiotoxicity was determined in studies with supraclinical concentrations of doxorubicin, and therefore may not represent the mechanism of action at physiological concentrations (reviewed in Minotti *et al.*, 2004). Indeed, a study examining mechanisms of action of doxorubicin in human myocardium have indicated that induced oxidative stress is involved in acute toxicity, whereas disruption in iron homeostatis is the predominant cause of long-term damage (Licata *et al.*, 2000). The concentrations used within our study were within published paediatric serum levels; therefore, alternative pathways could be involved. The timings selected within this study may not be appropriate for the study of oxidative stress; longer periods following exposure to chemotherapy agents may be required to observe any resulting oxidative stress. The time-course cultures of oxidative stress did not take into account ROS production timing, since ROS could not be fully assessed within prepubertal testis fragments due to the transient nature of these free radicals. Further cultures were therefore undertaken with a cell line representative of SSCs (GC-6spg) with utilization of fluorescent probes to further explore the potential role of ROS and oxidative stress in cisplatin, doxorubicin or vincristine gonadotoxicity.

5.4.4 Dose-dependent cytotoxicity of GC-6spg cell line following cisplatin, doxorubicin or vincristine exposure

Further experiments into the role of ROS production in chemotherapy-induced damage to the prepubertal testis were undertaken with the GC-6spg cell line, characteristics of SSCs, to determine ROS involvement in cisplatin-, doxorubicin- or vincristine-induced cytotoxicity. The move into cell line cultures was necessitated by the lack of effective techniques to assess ROS in tissue. The initial goal was to first optimize probes with use of cell culture line before moving into a tissue culture model where probes could be used to assess ROS production in a time-dependent manner. A dose-response was therefore required for cisplatin- (0.05-10µg/ml), doxorubicin- (0.05-0.5µg/ml) or vincristine-(0.0001-10µg/ml) induced cytotoxicity following 48hrs of drug exposure to enable selection of EC50 doses. Cytotoxicity was determined by

an MTT assay that measures cell viability through assessment of metabolic activity. A clear dose-response relationship was generated for cisplatin or doxorubicin treatment, with eventual complete loss of cell viability (no MTT signal) at the highest concentrations tested (cisplatin; 10µg/ml or doxorubicin; 0.5µg/ml). Vincristine treatment, however, produced a different dose-response curve since cell viability was maintained with ~50% viability at the highest concentration (10µg/ml). These findings confirm those of previously published works by Beaud *et al.* (2017), where dose- and time-dependent effects of vincristine (0.0009 - 10µg/ml) and/or doxorubicin (0.0005-5µg/ml) on the GC-6spg cell line after 24, 48 and 72hrs of exposure were examined. Significant reductions in cell viability (MTT signal) after 48hrs of drug exposure were observed at 0.1µg/ml vincristine (~50%) or 0.5µg/ml doxorubicin (no signal), generating similar dose-response curves as produced within this thesis. The Beaud *et al.* (2017) study further investigated the differences in the dose-responses of these drugs with annexin V and propidium iodide labelling. They found significant increases in the numbers of apoptotic and necrotic cells after 48hrs of vincristine treatment, whereas doxorubicin resulted in increased number of necrotic cells. From this finding, they hypothesised that the difference was due to a balance between cell death and cell proliferation upon vincristine treatment, compared to doxorubicin where the majority of cells were dead/necrotic. Additional experiments, for example BrdU uptake, would be required to test this hypothesis. EC50 doses for future experiments were chosen based upon the dose-response curves; doses that induced ~50% decrease in cell viability include 1 and 5µg/ml cisplatin, 0.2 and 0.4µg/ml doxorubicin or 0.01 and 0.5µg/ml vincristine. To validate the selections, the data was fit to a sigmoidal dose-response curve that calculated the EC50 doses on GraphPad. The doses for cisplatin (EC50 7µg/ml) and doxorubicin (EC50 0.2µ/ml) were similar or identical to those previously selected. A lower dose, however, of 0.001µg/ml was generated for vincristine. Since vincristine produced variable results over a wide range of concentrations, the highest known serum level of 0.5µg/ml was used for further experiments.

The time-course of apoptosis following cisplatin, doxorubicin or vincristine treatment in the GC-6spg cell line was assessed to confirm whether loss of cell viability shown in the dose-response studies was due to apoptosis or changes to metabolic activity. Additional analysis is required to examine to what extent reduced proliferation is involved in loss of cell viability. A fluorogenic probe that fluoresces upon activation of CC3/7 was added to culture and analysed at 6, 16, 24, 30, 40 and 48hrs post-exposure. Clear activation of apoptosis was observed for all chemotherapy agents. Comparisons between the three drugs is possible since concentrations of each drug reduced cell viability by ~50%. Cisplatin at the EC50 dose (5µg/ml) produced a stronger fluorescent signal by the 48hr time point, indicative of increased activation of CC3/7, compared to vincristine. Interestingly, the increase in CC3/7 following cisplatin treatment was apparent eight hrs before the loss in cell viability at the 48hr time point. This is in accordance with the data collected from Smart *et al.* (2018), with loss of germ cells eight hrs after increased CC3 expression in cisplatin-exposed tissues. In addition, doxorubicin treatment induced an early increase in CC3/7 expression, which may account for the lack of expression noted in Smart *et al.* (2018). The use of cell lines or primary cultures has previously been utilized to study the effects of chemotherapy agents to describe the toxicity and molecular targets of these drugs. Studies of cultured SSCs have shown reductions in cell viability, increased levels of apoptosis and enhanced expression of γH2AX, following cisplatin (Marcon *et al.*, 2010; Liu *et al.*, 2014; Shabani *et al.*, 2016), doxorubicin (Nurmio *et al.*, 2009b; Beaud *et al.*, 2017; Tremblay and Delbes, 2018) or vincristine (Marcon *et al.*, 2010; Beaud *et al.*, 2017). This thesis chapter aimed to extend our knowledge of the molecular targets of the studied chemotherapy agents by determining the role of ROS in the gonadotoxicity associated with these drugs.

5.4.5 Role of ROS in cisplatin-, doxorubicin- or vincristine-induced cytotoxicity to GC-6spg cell line

The fluorogenic probe, CellROX, was added to culture to analyse levels of ROS production upon cisplatin, doxorubicin or vincristine treatment. The fluorescent

signal generated by the CellROX, as well as the CellEVENT (CC3/7 activation) probe, was analysed with a confocal microscope at selected time points during the 48hr exposure window. From the experimental images, however, it was uncertain as to whether a comparison between the groups was applicable with this methodology. Therefore, it was not possible to appropriately assess the levels and timing of ROS production with these probes. The use of the CellROX probe (ROS) during a time-course study was problematic as it required a wash step before analysis to prevent any background signal. The same cells therefore could not be analysed over a long period, as with the CellEVENT probe. In addition, washing of the wells to remove the probe would result in loss of cells that had detached from the bottom upon cell death. This therefore would result in less cells attached to the well, making it harder to compare between the experimental conditions. Initial images acquired with the confocal microscope were taken with the assessor deciding the focal plane that the images would be taken from. However, upon slightly changing the confocal plane, the levels of expression varied. To remove assessor bias, a focal range along the Z-axis was set when procuring further images that were stitched together. A positive signal was clear in the control and vincristine treated cells at the 24hr time point. Vincristine is considered to induce low levels of oxidative stress with one study from the 1980s indicating a role in glutathione metabolism leading to oxidative stress (Beck, 1980 as descibed in Jordan *et al.*, 1991). ROS following vincristine treatment may occur upon activation of apoptosis due to cytochrome C release from mitochondria, a consequence rather than stimulator of apoptosis (reviewed in Gorrini *et al.*, 2013). In regards to the positive signal in the control, the production of ROS is a normal physiological process, therefore it would be expected that untreated cells would produce ROS to an extent. Analysing these images for example through counting the number of positive CellROX cells per an area or total cell number would not be suitable. If images were to be analysed it would necessitate assessment of the intensity of the signal produced, which would require an observable difference in intensity levels after chemotherapy treatment. Measurement of the intensity of a signal has been performed by Smart *et al.* (2018) on ImageJ with γH2AX expression

in cultured prepubertal testis fragments. The positive signal noted with vincristine (0.5µg/ml) treatment may also be a result of using a concentration of vincristine that is too high, with the lack of signal in cisplatin (1µg/ml) or doxorubicin (0.1µg/ml) exposed cells due to concentrations being too low. Future experiments therefore should include cells exposed to cisplatin at 5µg/ml, doxorubicin 0.2µg/ml or vincristine at 0.001µg/ml.

The CellROX probe has previously been used to assess ROS production in two cell lines following doxorubicin treatment; the SSC representative GC-6spg and immature Sertoli cell Ser-W3 cell line (Tremblay and Delbes, 2018). The fluorescent signal generated upon ROS production was measured with use of microplate reader at selected time points. This study concluded that ROS, and oxidative stress as shown by 8-OHdG, was not involved in cytotoxicity to the GC-6spg cell line. Alternatively, ROS production increased in the Ser-W3 cell line with concomitant increase in 8-OHdG levels. The Ser-W3 cell line was more sensitive to the doxorubicin, as measured by MTT assay, compared to the GC-6spg cell line. Tremblay and Delbes, (2018) discuss that this result may be due to increased proliferation of the Sertoli cell line which is not physiological (Nurmio *et al.*, 2009b; Smart *et al.*, 2018). A rise in proliferation would also result in enhanced production of ROS due to mitochondrial activity, indeed ROS production in their vehicle treated controls increases with culture, although this was not significant. The CellROX probe was chosen to investigate ROS in this thesis as it has been used in combination with the CellEVENT probe. The fluorescent signal was analysed through use of confocal microscope, different methodologies to detect the CellROX signal may be more appropriate and include use of flow cytometry or high content screening. Additionally, there are other methods of detecting ROS species production *in vitro* that could be used instead of the CellROX probe. These methods normally rely on fluorescence or chemiluminescence to detect global ROS/RNS production or more specific free radicals, however, the specificity of many of these reagents can be argued (reviewed in Pavelescu, 2015). More commonly used probes include; dihydroethidium that

fluoresces upon oxidation by superoxide radicals, 2'-7' dichlorofluorescin (H_2DCF) which is converted to 2'-7'dichlorofluorescein (DCF) through action of hydrogen peroxide to produce a fluorescent signal (reviewed in Held and BioTek Instruments, 2015; Griendling *et al.*, 2016).

5.4.7 Concluding Remarks

To conclude, this chapter set out to explore the role of ROS and oxidative stress in cisplatin-, doxorubicin- or vincristine-induced gonadotoxicity to the mouse prepubertal testis and rat G6-spg cell line. A dose-response relationship for each drug in the different cultures systems was first determined to select toxic concentrations to investigate ROS or oxidative stress. Oxidative stress was measured in prepubertal testis tissue; however, based on the immunohistochemistry results, there is no evidence of lipid peroxidation or DNA/RNA damage following treatment. Production of ROS was assessed in the GC-6spg cell line but could not be appropriately analysed when using a confocal microscope. The role of ROS and oxidative stress, therefore, needs further investigation with alternative approaches/methodologies as outlined in the above discussion. There is, however, the possibility that ROS is not involved in the gonadotoxicity/cytotoxicity of cisplatin or doxorubicin treatment. This was the conclusion regarding doxorubicin-induced cytotoxicity to GC-6spg cell line following use of CellROX probe in a previous study, with authors suggesting GC-6spg cells/spermatogonia may be more resistant to oxidative stress due to the presence of antioxidant Cu/Zn-superoxide dismutase and/or reduced mitochondrial activity (Celino *et al.*, 2011; Guo *et al.*, 2017; Tremblay and Delbes, 2018). Another approach to understanding the role of ROS and oxidative stress involves testing out a range of antioxidants to determine whether they can protect against chemotherapy-induced damage. This is further investigated in Chapter 6 of this thesis, where protection from antioxidants would suggest a role of ROS and oxidative stress in cisplatin-, doxorubicin- or vincristine-induced gonadotoxicity/cytotoxicity to mouse prepubertal testis or rat GC-6spg cell line.

Chapter 6. Protective effects of Antioxidants

6.1 Introduction

Antioxidants play a crucial role in cellular defence against ROS/RNS, maintaining redox equilibrium of cells/tissues (reviewed in Aitken and Roman, 2008). The role of oxidative stress in cancer is complex, with cancerous cells generally exhibiting higher levels of oxidative stress than normal, healthy tissues, as discussed in Chapter 5. Certain chemotherapy agents induce oxidative stress as an underlying mechanism of action, resulting in cell death of cancerous cells and potentially damaging healthy tissues as a side effect (reviewed in Conklin, 2004). Antioxidant therapy during cancer treatment may therefore be an effective strategy to protect healthy tissues from excessive oxidative stress induced by the chemotherapy drugs, without interfering with the ability of the drugs to kill off the cancerous cells. The idea of including antioxidants during cancer treatment to protect against chemotherapy-induced damage to healthy tissues is a controversial subject area (reviewed in Khurana *et al.*, 2018). To date, there have been a number of clinical studies and animal model studies investigating the impact of antioxidants as an adjuvant to cancer treatment, with those debating for and against inclusion (reviewed in Block *et al.*, 2008; Yasueda *et al.*, 2016; Singh *et al.*, 2018). This chapter will explore the potential of antioxidants to protect against chemotherapy-induced damage to the mouse prepubertal testis and rat GC-6spg cell line. To date, the antioxidants amifostine, cartinine, vitamin C, curcumin, BM-MSCs and certain herbal extracts have been investigated in relation to the protective effects against cisplatin and/or doxorubicin in the prepubertal testis/cells (see Chapter 1, Table 1.9). The antioxidants examined in this thesis include melatonin, n-acetylcysteine (NAC) and lycopene.

6.1.2 Antioxidant Defence Systems

6.1.2.1 Overview

Antioxidants have been defined in Halliwell *et al.* (2015), a book written by key researchers in the field since the 1980s, as **"any substance that, when present at low**

concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". In other words, antioxidants are stable molecules/compounds that prevent or limit the damage induced by free radicals (reviewed in Lobo *et al.*, 2010). An imbalance in antioxidants and free radical production will ultimately result in oxidative or nitrosative stress; this may be due to over production of ROS/RNS that overwhelms the antioxidant defence system, or due to a reduction in antioxidant levels and/or function. Antioxidants have a role in maintaining the redox state by neutralising free radical species through different actions depending on the free radical, and includes the antioxidant acting as a chain-breaking antioxidant, radical species scavenger, electron donor, hydrogen donor, singlet oxygen quencher, peroxide decomposer, enzyme inhibitor and metal-chelating agent (reviewed in Lobo *et al.*, 2010). Antioxidants can be broadly classified as enzymatic or non-enzymatic, as well as endogenous or exogenous (Table 6.1) (reviewed in Pham-Huy *et al.*, 2008; Lobo *et al.*, 2010; Birben *et al.*, 2012). There are a number of antioxidants within cells/tissues that can work synergistically to protect against oxidative/nitrosative stress, with cells often having a specific arsenal of antioxidants. Antioxidants can exist in cells/tissues/plasma in a lipid state (Vit E, flavonoids), as well as an aqueous phase (Vit C, uric acid) (reviewed in Young and Woodside, 2001). Certain antioxidants are capable of redox cycling whereas other are consumed during the antioxidant action, for example melatonin and vitamins, and need to be replenished. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione enzymes that act to detoxify superoxide radicals, forming hydrogen peroxide and ultimately water (Fig 6.1). Non-enzymatic antioxidants include endogenous antioxidants, produced as a by-product of cellular metabolism, or exogenous antioxidants that are obtained from our diet/supplements. Examples of endogenous, non-enzymatic antioxidants include L-arginine, coenzyme Q10, melatonin and metal binding proteins. Non-enzymatic, exogenous antioxidants are acquired through a diet rich in antioxidant containing foods such as fruits and vegetables, or through use of dietary supplements, examples include carotenes, flavonoids, trace metals (selenium, zinc, manganese) and vitamins

(reviewed in Rice-Evans and Miller, 1995; Pham-Huy *et al.*, 2008; Huang, 2018). Use of dietary supplements is increasing with health benefits noted when such supplements are taken at the recommended dose, however, negative effects on health are apparent upon excessive use (Bouayed and Bohn, 2010). Overall, there is a complex, finely tuned system of antioxidants that the body can use to protect from excessive free radical production.

Table 6.1 Examples of enzymatic and non-enzymatic antioxidants. Information from Yasueda *et al.* (2016). Highlighted in green are antioxidants investigated in this chapter

Enzymatic	Non-Enzymatic
Superoxide dismutase (SOD) Catalase (CAT) Glutathione peroxidase (GTPx) Thioredoxin (TRX) Peroxiredoxin (PRX) Glutathione transferase (GSP) Glutathione reductase (GR)	Vitamins e.g. Vit A (retinol), biquinone (Vit Q, coenzyme Q10), tocopherol (Vit E) & ascorbic acid (Vit C) Carotenoids e.g. α-Carotene, β-Carotene, **lycopene** & lutein Phytochemicals e.g. **melatonin** & α-Lipoic acid Polyphenols e.g. curcumin, ellagic acid, isoflavane & Lignan Amino acids and related substances e.g. glutathione, **n-acetylcysteine**, L-arginine & albumin Minerals e.g. selenium, chorium, zinc & manganese

6.1.1.2 Antioxidants in the Testis

Within the testis, an intricate antioxidant defence system utilizes both enzymatic and non-enzymatic antioxidants to protect against oxidative stress: this topic has been reviewed extensively for example by Aitken and Roman, (2008) and Asadi *et al.* (2017) with focus of multiple reviews on male infertility (Bisht *et al.*, 2017; Aitken, 2020;

Barati *et al.*, 2020). This is due to the susceptibility of the testis and mature spermatozoa, as discussed in Chapter 5, to high levels of oxidative stress owing to the high-energy demands of spermatogenesis/steroidogenesis and overall structure of germ cells/spermatozoa. The testis utilizes enzymatic antioxidants including SOD and glutathione enzymes (peroxidase and transferase), with CAT not heavily involved in the testis antioxidant defences (overview in Fig 6.1) (reviewed in Aitken and Roman, 2008). SOD is the first line of defence against free radical production and is an important antioxidant in the testis since all three isoforms are expressed in the adult; including the conventional cytosolic Cu/Zn SOD, mitochondrial Fe/Mn SOD and the cell specific form of extracellular SOD (reviewed in Mruk *et al.*, 2002). Multiple isoforms of glutathione peroxidase are also present in the adult testis including cellular and plasma glutathione peroxidases, as well as phospholipid hydroperoxide glutathione peroxidase that acts upon lipid peroxides in the cellular membrane (reviewed in Aitken and Roman, 2008). The expression and activity of these enzymatic antioxidants are differentially expressed during testis development and spermatogenesis. Studies from animal models have compared the activity and mRNA levels of these antioxidants from tissue lysate and/or isolated germ cells from prepubertal and adult rodents. In the prepubertal testis, activity of SOD is high with expression of Cu/Zn SOD mRNA, whereas the activity of glutathione peroxidases is low with expression of cellular glutathione peroxidase mRNA (Peltola *et al.*, 1992; Gu *et al.*, 1995). During spermatogenesis, the Cu/Zn SOD is highly expressed in spermatogonia, as shown in Japanese eels (Celino *et al.*, 2011). With increasing age, glutathione transferase activity rises with mRNA expression levels and activity of cellular glutathione peroxidases and levels of glutathione. Fe/Mn SOD and phospholipid hydroperoxide glutathione peroxidase mRNA are expressed at the onset of spermatogenesis and in adulthood, with expression mainly found in meiotic and post-meiotic germ cells (Peltola *et al.*, 1992; Gu and Hecht, 1996; Zini and Schlegel, 1997; Baek *et al.*, 2007). The cellular localisation of these antioxidants differ with reported increased activity of glutathione enzymes in somatic Sertoli cells compared to higher SOD activities and glutathione levels in spermatocytes,

spermatids and spermatozoa (Den Boer *et al.*, 1989; Bauché *et al.*, 1994; Kaneko *et al.*, 2002). The extracellular SOD is unusual with expression limited to certain tissues but is expressed in germ and Sertoli cells, particularly in the latter (reviewed in Mruk *et al.*, 2002). In addition to the above enzymatic antioxidants, there are also a number of non-enzymatic antioxidants involved in the antioxidant defence system of the testis. These include both endogenous and exogenous antioxidants with low molecular weight such as zinc, selenium, vitamins C and E, melatonin and cytochrome C (reviewed in Aitken and Roman, 2008; Asadi *et al.*, 2017). Indeed, the testis has a specific form of cytochrome C restricted to later stages of spermatogenesis (Goldberg *et al.*, 1977; Narisawa *et al.*, 2002).

Enzymatic antioxidant defence systems

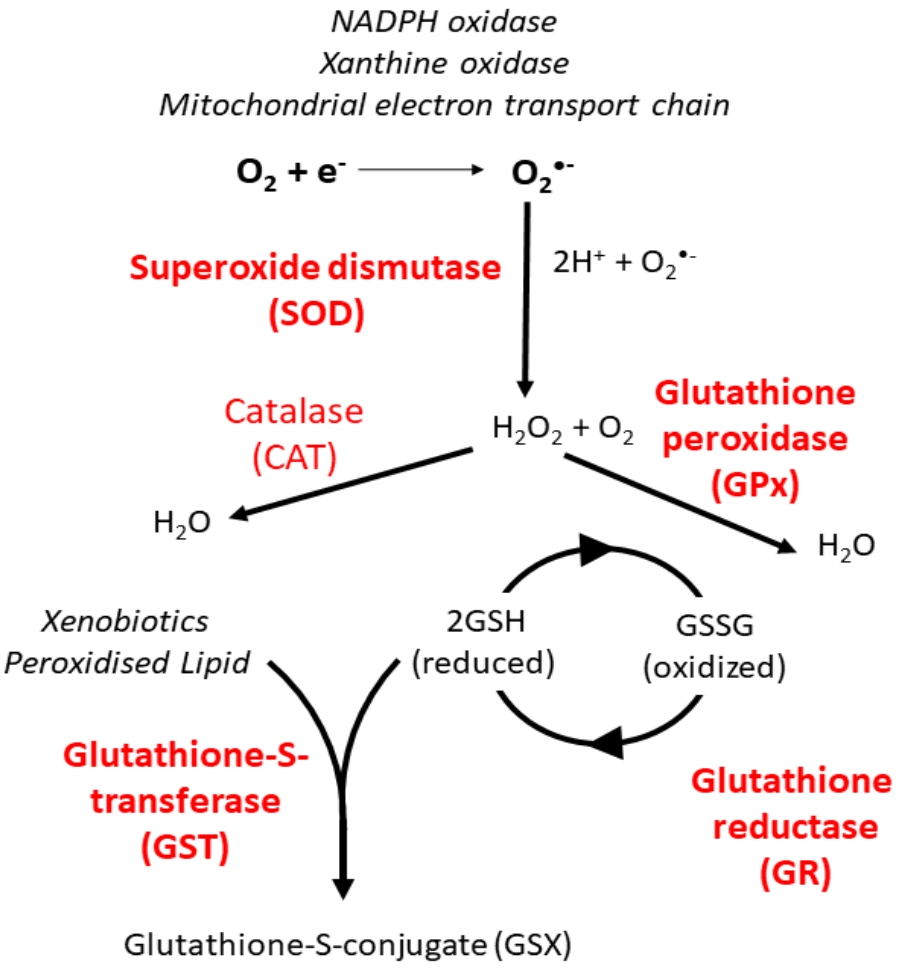


Figure 6.1: Enzymatic pathway for antioxidant defence against free radial species. The superoxide radical is produced through multiple different cellular activities and is converted into hydrogen peroxide through the action of superoxide dismutase enzymes (SOD). The hydrogen peroxide is rapidly converted to water by the action of catalse (CAT) or glutathione peroxidases (GPx) enzymes. CAT is not heavily invovled in protecting the testis. The GPx protein uses reduced glutathione (GSH) as an electron donor to convert the hydrogen peroxide into water, producing oxidized glutathione (GSSG). The glutathione reductase (GR) enzyme, in the presence of NADPH, catalyzes the reduction of one molar equlivanet of GSSG back to the reduced forming two molar equivalents GSH. Glutathione-S-transferase (GST) enzyme is involved in detoxification of xenobiotics and lipid peroxidation products by conugating these substance to reduced glutathione (GSH) which are then excreted from the cell as glutathione-S-conjugate. Image modified from Aitken and Roman, (2008). Red indicates enzyme.

6.1.3 Antioxidants to Protect Against Chemotherapy-Induced Damage

The use of antioxidants during cancer treatment is a contentious issue with those advocating 'for' and 'against' inclusion, with multiple reviews assessing data from clinical and animal model studies (Block *et al.*, 2008; Yasueda *et al.*, 2016; Khurana *et al.*, 2018; Singh *et al.*, 2018). For example, the most recent systematic review assessed 93 clinical, 56 animal model and 35 *in vitro* studies covering the impact of 13 antioxidants (Singh *et al.*, 2018). Those advocating 'for' use of antioxidants as an adjuvant to chemotherapy treatment state that such antioxidants can prevent oxidative stress related toxic side effects of chemotherapy treatment on healthy tissues, therefore increasing survival rate and effectiveness of chemotherapy since patients can tolerate higher doses of drugs (reviewed in Moss, 2006; Simone *et al.*, 2007a, 2007b; Singh *et al.*, 2018). Those that reason 'against' state inclusion will interfere with the chemotherapy treatment, and potentially attribute to progression of the cancer and reduce survival rate (reviewed in D'Andrea, 2005; Lawenda *et al.*, 2008). Conclusions are hard to draw from the current data since comparisons between studies is difficult due to differences in study designs, cancer types, ages, chemotherapy treatment regimens, antioxidant usage and concentrations, making it difficult to perform a meta-analysis of the studies (reviewed in Nakayama *et al.*, 2011; Yasueda *et al.*, 2016; Singh *et al.*, 2018). Overall, whether antioxidants are beneficial or detrimental as an adjuvant to treatment is unclear and further well-controlled, powerful clinical research trials are needed (reviewed in Conklin and Conklin, 2000; D'Andrea, 2005; Block *et al.*, 2008; Nakayama *et al.*, 2011; Fuchs-Tarlovsky, 2013; Yasueda *et al.*, 2016; Khurana *et al.*, 2018). Cancer patients report increased usage of vitamins and mineral dietary supplements with estimates between 13 and 87%, with use varying with cancer type, age and education (reviewed in Block *et al.*, 2008). A study from the US indicates that between 14 and 32% of patients initiate use of dietary supplements after cancer diagnosis and that around 70% of their doctors are unaware that their patients are taking these supplements during their cancer treatment (Velicer and Ulrich, 2008).

Within this thesis, the protective effects of melatonin, NAC and lycopene were investigated for their protective effects against cisplatin-, doxorubicin- or vincristine-induced damage. These antioxidants were chosen since protective effects against chemotherapy treatments have been noted upon review (see below), with lycopene chosen due to the recent publication of the results of a clinical trial indicating a supplementation improved semen quality in healthy men (Williams *et al.*, 2020). The compounds were also chosen as they are each from a different class/group of antioxidants, and will therefore potentially have different underlying mechanisms of action. For example melatonin is considered a phytochemical, NAC an amino acid substance and lycopene a carotenoid (reviewed in Yasueda *et al.*, 2016, see table 6.1).

6.1.3.1 Melatonin

Melatonin (5-methoxytryptamine) is a neurohormone secreted from the pineal body during periods of darkness that regulates circadian rhythms such as sleep wake cycles, body temperature and neuroendocrine rhythm (reviewed in Tordjman *et al.*, 2017). For example, melatonin can regulate activity of the HPG axis to control release of gonadotrophins in seasonal breeders. Melatonin is a lipid soluble compound that can act as a potent antioxidant since it contains two free electrons that can quench free radicals, rather than one, with metabolites also displaying antioxidant activity (reviewed in Tan *et al.*, 2005). Melatonin can also exert its effects through binding to melatonin G protein-coupled receptors (MT1 and MT2), which are expressed in the testis of rodents, particularly Leydig cells, during development and in the adult (Drew *et al.*, 2001; Izzo *et al.*, 2010). The testis itself is also reported to secrete melatonin, as shown in the rodent testis through assessment of key enzymes involved in biosynthesis (Tijmes *et al.*, 1996; Stefulj *et al.*, 2001). Within the testis, melatonin is proposed to play a role in steroidogenesis, spermatogenesis and testicular function through reported action on the somatic cell population (reviewed in Frungieri *et al.*, 2017). Indeed, melatonin treatment has been shown to decrease testosterone production, enhance sensitivity of Sertoli cells to FSH and increase the metabolic

activity of Sertoli cells in animal model studies (Heindel *et al.*, 1984; Kuş *et al.*, 2002; Rocha *et al.*, 2014; Qin *et al.*, 2015). The involvement of melatonin in male fertility is not well understood, however levels of melatonin have been shown to be reduced in men with fertility issues, with reported roles in protection against oxidative stress and inflammation (Awad *et al.*, 2006; Rossi *et al.*, 2014). Additional roles of melatonin as a cytoprotectant for testicular tissue pieces for fertility preservation, stimulant for mouse SSC proliferation and differentiation (Sun *et al.*, 2020), as well an anti-cancer role have also been proposed (reviewed in Di Bella *et al.*, 2013). The protective effects of melatonin have also be investigated in relation to other pathologies of the testis that may involve oxidative stress, such heat-shock (Zhang *et al.*, 2020). Review of the literature assessing the inclusion of melatonin in cancer treatments found that in a majority of the studies there were no adverse effects following inclusion, with reporting of reduced chemotherapy-induced toxicities such as myelosuppression and neurotoxicity (reviewed in Block *et al.*, 2008; Yasueda *et al.*, 2016). Following supplementation in adults, serum levels of melatonin can vary from 400-4000pg/ml, depending on the dose (0.4 – 4mg), which is normally given at night (Gooneratne *et al.*, 2012; Mistraletti *et al.*, 2017).

6.1.3.2 N-acetylcysteine (NAC)

NAC is an amino acid related drug that is a precursor of L-cysteine, which stimulates production of glutathione (reduced GSH), enhances detoxification and also directly acts as an antioxidant to scavenge free radical species. This drug is well tolerated and is used in the treatment of paracetamol overdose, polycystic ovarian syndrome, preterm birth and asthma to name a few (reviewed in Mokhtari *et al.*, 2017). Systematic reviews have assessed studies investigating side effects of NAC inclusion and have found no indication of adverse side effects upon treatment in a small number of studies, with noted reduced chemotherapy-induced cardiotoxicity or neurotoxicity in two separate studies (reviewed in Block *et al.*, 2008; Nakayama *et al.*, 2011; Yasueda *et al.*, 2016). Serum levels following supplementation in adults

range from 0.35-20.8µg/ml, depending on dose administered (200-800mg/m^2) (Holdiness, 1991; Pendyala and Creaven, 1995).

6.1.3.3 Lycopene

Lycopene is a carotene obtained from consuming fruits including tomatoes, watermelons and guava. Lycopene is a potent free radical scavenger of singlet oxygen and peroxyl radicals as well as an immunomodulator/anti-inflammatory agent. The antioxidant capacity of lycopene is high and has reported enhanced scavenger activity compared to vitamin E (100X) and glutathione (125X) (reviewed in Kumar *et al.*, 2017). Lycopene serum levels can range from 0.07-1.04µg/ml, which have been reported after consumption of tomato juice/tomato products in adults (Jenab *et al.*, 2005; Datta *et al.*, 2013).

6.1.4 Aims and Hypothesis

This chapter aims to investigate the protective effects of the antioxidants melatonin, NAC and lycopene against cisplatin, doxorubicin or vincristine exposure in mouse prepubertal testis tissue and rat GC-6spg cell line. Cisplatin and doxorubicin are considered to induce damage through generating oxidative stress, therefore it was hypothesised that antioxidant co-treatment would protect the germ cell population from chemotherapy-induced damage. On the other hand, since vincristine is not known to induce oxidative stress as a mechanism of action, it was hypothesised that antioxidant co-treatment would not protect against vincristine-induced damage to the prepubertal testis/GC-6spg cell line, if any damage did occur. Vincristine was included in these studies as a 'negative control' chemotherapy drug, as in Chapter 5.

6.2 Methods

6.2.1 Experiment 1: Determining protective effects of antioxidants against chemotherapy-induced damage to GC-6spg cell line

The antioxidants melatonin, NAC and lycopene were tested to examine their protective capacity against cisplatin-, doxorubicin- or vincristine-induced damage to the GC-6spg cell line. Cytotoxicity was assessed by an MTT assay to calculate the cell viability following co-treatment with chemotherapy agents and the selected antioxidants for 48hrs (see 2.5 for culture conditions and 2.7 for MTT assay). Calculated IC50 doses of the chemotherapy agents were determined in Chapter 5 (see 5.3.1) with 5µg/ml cisplatin, 0.2µg/ml doxorubicin or 0.5µg/ml vincristine used in co-treatment experiments.

The antioxidants were purchased in powder form with stock concentrations prepared for each experiment in a similar manner to powder forms of chemotherapy agents (see Chapter 2.2.1). Melatonin (M5250, Sigma) was dissolved in ethanol (20821.330, VWR) to produce 20mg/ml stock solutions. NAC (A7250, Sigma) was made up in deionized water (15230089, ThermoFisher) to produce a 100mg/ml stock solutions; the solution was heated at 37^{o}C in a water bath to facilitate dissolving. Lycopene (SMB00706, Sigma) was dissolved in DMSO (D2438, Sigma) producing a 2mg/ml stock solution. Vortexing aided in dissolving the antioxidants within the selected solvents. Concentrations of antioxidants tested were determined by examining the literature and serum levels after supplementation. Concentrations examined include 0.0002-20µg/ml melatonin, 0.1-1000µg/ml NAC and 0.0001-10µg/ml lycopene. For each experiment, a dose-response for the antioxidant alone was included to rule out any potential negative effects on cell viability in the GC-6spg cell line. In addition, 'positive controls' were included where cells were exposed to the chemotherapy agents at cytotoxic doses to confirm the effects of the drugs on cell viability and to compare to the co-treated cells to determine protective capacity with antioxidant treatment. GC-6spg cells were co-treated with a range of concentrations of each antioxidant and either 5µg/ml cisplatin, 0.2µg/ml doxorubicin or 0.5µg/ml vincristine. Clinical

preparations of doxorubicin and cisplatin were utilized, whereas the powder form of vincristine was dissolved in PBS. Equivalent volumes of antioxidant solvent and/or PBS were added to the wells to ensure equal concentrations of solvents across the treatment groups. Controls cells were therefore exposed to equivalent concentrations of antioxidant solvent and PBS. Melatonin and NAC experiments were repeated four times and lycopene twice.

6.2.2 Experiment 2: Time-course of CC3/7 expression with antioxidants co-treatment

To determine whether antioxidant co-treatment had an effect on chemotherapy-induced apoptosis, an additional experiment was undertaken to measure activation of CC3/7 following NAC (0.1, 100 or 1000µg/ml) co-treatment with cisplatin (5µg/ml) or vincristine (0.5µg/ml). The GC-6spg cell line was cultured and NAC/cisplatin or vincristine added as described above with the addition of the CellEVENT probe (2µM) (see 2.7). The fluorescence of the cells were measured at 24 and 48hrs post-exposure with a microplate reader. An MTT assay was performed at the end of the 48hrs to confirm cell viabilities. These experiments were interrupted by Covid-19 and were therefore only performed once: as such, they were not analysed statistically.

6.2.3 Experiment 3: Determining protective effects of NAC against chemotherapy-induced damage to prepubertal mouse testis

The effects of co-treatment of NAC with cisplatin or vincristine were analysed with cultured prepubertal testis fragments to confirm preliminary findings from cell culture work. Stock concentrations of NAC were prepared as described above (6.2.1), with 5 or 100µg/ml NAC added to cultures using the same methodology as described in relation to chemotherapy agents (2.2.1). Clinical preparations of cisplatin and powdered vincristine dissolved in PBS were used within this experiment at working concentrations of 0.5µg/ml cisplatin or 0.1µg/ml vincristine. Tissues were cultured as previously described, with tissues exposed to antioxidant/chemotherapy on the second day of culture for 24hrs (2.1). Tissues were exposed to either; control

conditions, NAC, chemotherapy agent or co-treated with NAC and chemotherapy agent. Equivalent volumes of antioxidant solvent and/or PBS were added to the wells to ensure equal concentrations of solvents across the treatment groups; therefore, controls were exposed to PBS and deionized water. Immunofluorescence was performed with the number of non-proliferating (MVH+, BrdU-) and proliferating (MVH+. BrdU+) germ cells manually counted with density expressed by area of tissue section (see 2.4). A sample size of 'n'=4 from four culture runs were set up with tissues fixed and ready for analysis, however, due to Covid-19 only an 'n'=1 for these experiments was processed for histology, therefore statistical analysis could not be performed.

6.2.4 Statistics

Due to low 'n' number for experiments two (6.2.2) and three (6.2.3), only the data collected from experiment one (6.2.1) was statistically analysed. Originally, the data was to be analysed with a Two-way ANOVA with the different drugs and concentrations of antioxidants considered two independent factors that would be assessed for interactions. Normality was tested as per Chapter 2.9 with data found to not follow a normal distribution (Appendix C Figure 4). Performing a Two-way ANOVA would therefore not be appropriate since normality is an assumption of this test. Transformation failed to normalise the data to enable statistical analysis with a parametric test. An equivalent non-parametric two-way ANOVA test, the Friedman's test, could not be performed due to issues with access to the software to perform this test (due to Covid-19). An alternative approach was therefore undertaken in order to analyse these data statistically. The data was separated for each drug/antioxidant and normal distribution re-analysed Appendix C Figure 5 & 6. The dose-response for the antioxidant alone was statistically analysed with a one-sample t test (parametric) or one-sample Wilcoxon test (nonparametric) as described in Chapter 2.9, where a $p < 0.05$ deemed the antioxidant to be statistically significant from the hypothetical control 100% viability. In addition, one-way ANOVA/Kruskall-Wallis and post-hoc tests were used to compare the antioxidant/chemotherapy co-

treated cells with the cells exposed to chemotherapy treatment alone to determine whether the presence of the antioxidant altered cell viability (see Chapter 2.9).

6.3 Results

6.3.1 Melatonin partially protects against cisplatin-induced damage to the GC-6spg cell line

The protective capacity of the antioxidant melatonin against cisplatin-, doxorubicin- or vincristine-induced damage to the GC-6spg cell line was determined following 48hrs of co-treatment by MTT assay to assess cell viability. A wide range of melatonin concentrations (0.0002 to 20µg/ml) were tested against cytotoxic doses of cisplatin (5µg/ml), doxorubicin (0.2µg/ml) or vincristine (0.5µg/ml).

The impact to the GC-6spg cell line following exposure to melatonin alone produced variable results with cell viabilities varying dependent on culture run (Fig 6.2A). For each concentration tested, there were a wide range of cell viabilities from the four culture runs, ultimately resulting in large SEMs and 95% confidence intervals for the calculated means. For example, exposure to 2µg/ml melatonin resulted in cell viability of 97 +/- 21% with a 95% confidence interval (95% CI) of 30%, 166% (widest example of confidence interval). These data were analysed with a one-sample t test to compare the mean cell viabilities from melatonin treated cells to a 100% cell viability, calculated from the control wells (control shown as red dotted in line in Fig 6.2). This method of statistical analysis would therefore not take into account the spread of the data collected to calculate the mean. There was no significant difference between the mean of the melatonin treated cells compared to the control 100% cell viability for all concentrations of melatonin tested ('n'=4, $p > 0.05$; Fig 6.2A). There were, however, large discrepancies between the mean melatonin treated cell viability and 100% control viability for the three lower concentrations of 0.0002µg/ml (discrepancy 30 +/- 14%), 0.002µg/ml (23 +/- 11%) and 0.02µg/ml (21 +/- 17%) melatonin, which were not statistically significant. Additional cultures are required

before firm conclusions can be made as to whether melatonin itself has an effect on cell viability of the GC-6spg cell line.

The cell viabilities for melatonin co-treated cells with cisplatin, doxorubicin or vincristine were compared to the effects of the chemotherapy drug alone to determine whether melatonin was protective against the chemotherapy-induced damage. There was a significant protective effect of melatonin 20µg/ml co-treatment against cisplatin-induced damage ('n'=4, p=0.009), cell viability following cisplatin treatment reduced to 26 +/- 4% (95% CI; 13%, 38%) whereas co-treatment was less damaging with 52 +/- 3% (95% CI; 42%, 62%) of cells remaining viable (Fig 6.2 Bi). There was no significant protective effect of melatonin co-treatment with doxorubicin or vincristine treatment ('n'=4, $p > 0.05$ for each drug) (Fig 6.2 Bii, iii). A pattern of higher cell viabilities for culture one was noted for the higher concentrations of melatonin co-treatment with doxorubicin or vincristine, this trend however was not observed for cisplatin where protective effects were noted.

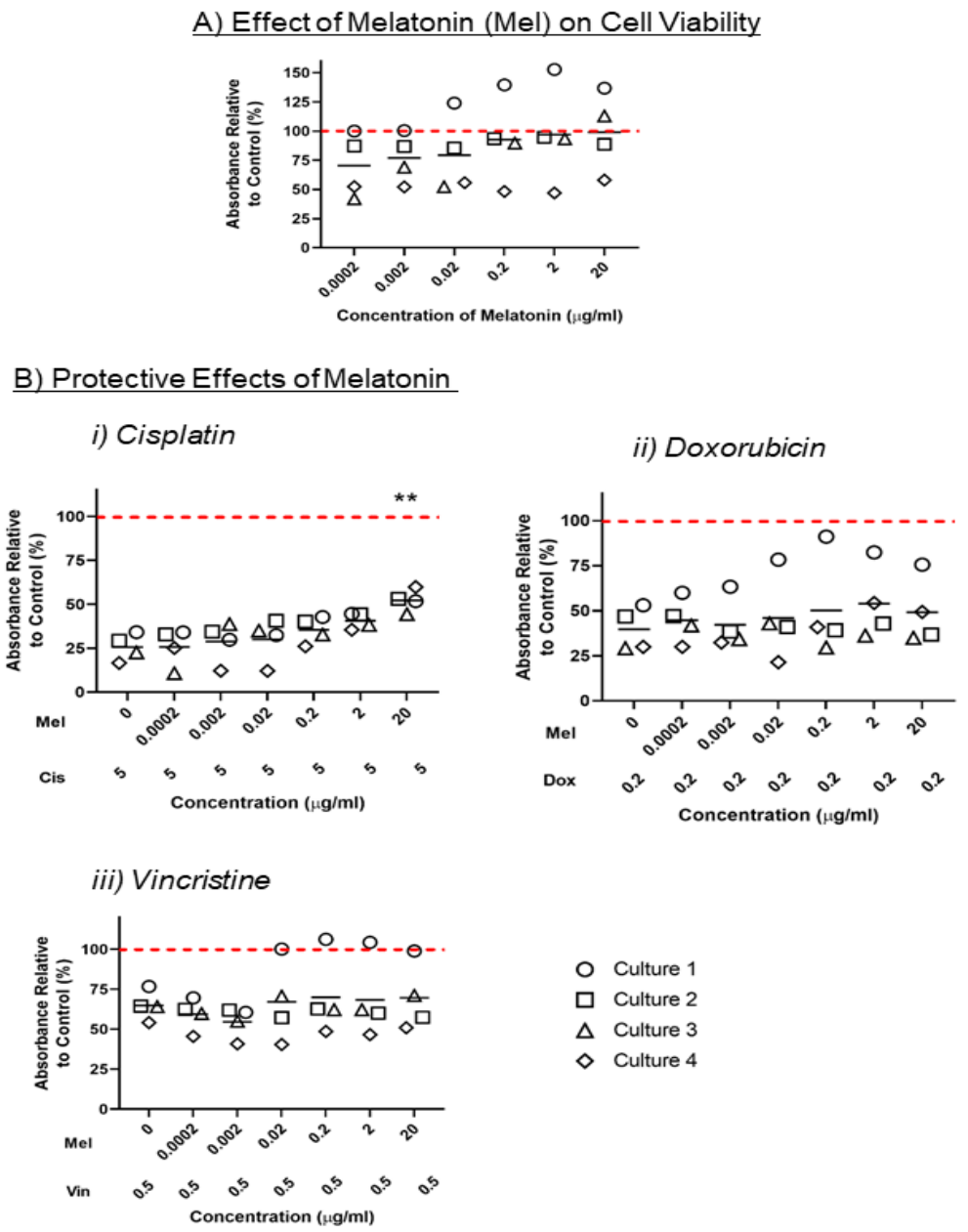


Figure 6.2 Effects of melatonin co-treatment on chemotherapy-induced damage to GC-6spg cell line. A) The impact of melatonin (0.0002-20µg/ml) on the GC-6spg cell line**.** B) Protective effects of melatonin against i) cisplatin-induced cytotoxicity (5µg/ml), whereas melatonin did not ameliorate effects of ii) doxorubicin (0.2µg/ml) or iii) vincristine (0.5µg/ml) cytotoxicity. Red line represents the cell viability of control cells at 100%. See Appendix C Figure 5 for QQ-plots analysing normal distribution. A) One-sample t test to compare mean cell viabilities of melatonin-exposed cells to 100% cell viability. B) Kruskall Wallis and Dunn's multiple comparisons post-hoc test to compare cell viabilities of co-treated cells to drug exposed cells. **p<0.01. 'n'=4 with data shown in relation to individual culture runs with grand mean.

6.3.2. N-acetylcysteine (NAC) co-treatment has differential effects with cisplatin or vincristine treatment of the GC-6spg cell line

To determine the protective effects of NAC, GC-6spg cells were co-treated with NAC (0.1-1000µg/ml) and either cisplatin (5µg/ml), doxorubicin (0.2µg/ml) or vincristine (0.5µg/ml) for 48hrs and cell viabilities determined by an MTT assay. NAC treatment alone did not have a significant effect on cell viability compared to 100% control ('n'=4, $p > 0.05$) (Fig 6.3 A). For example, 1000µg/ml NAC treated cells were 88 +/- 11% (95% CI 52%, 124%) viable, which resulted in a discrepancy of 12 +/- 11% from the 100% control value and was not statistically significant ('n'=4, $p > 0.05$). Co-treatment of cisplatin-exposed cells with NAC at 100 or 1000µg/ml partially protected the cells from cisplatin-induced damage ('n'=4, $p < 0.05$ for both) (Fig 6.3 Bi). Cisplatin treatment reduced cell viability to 28 +/- 7%, whereas co-treatment with NAC increased the cell viability to 65 +/- 11% and 67 +/- 12% respectively for 100µg/ml and 1000µg/ml NAC. There was no protective effect of NAC co-treatment with doxorubicin for all concentrations of NAC tested ('n'=4, $p > 0.05$) (Fig 6.3 Bii). Surprisingly, a negative effect of NAC co-treatment with vincristine was observed with increasing concentrations of NAC ('n'=4, $p < 0.05$) (Fig 6.3 Biii). Co-treatment with 1000µg/ml of NAC with vincristine reduced cell viability of the GC-6spg cell line to 12 +/- 1% compared to vincristine alone, whereby 73 +/- 7% of cells were viable after treatment ('n'=4, $p=0.002$).

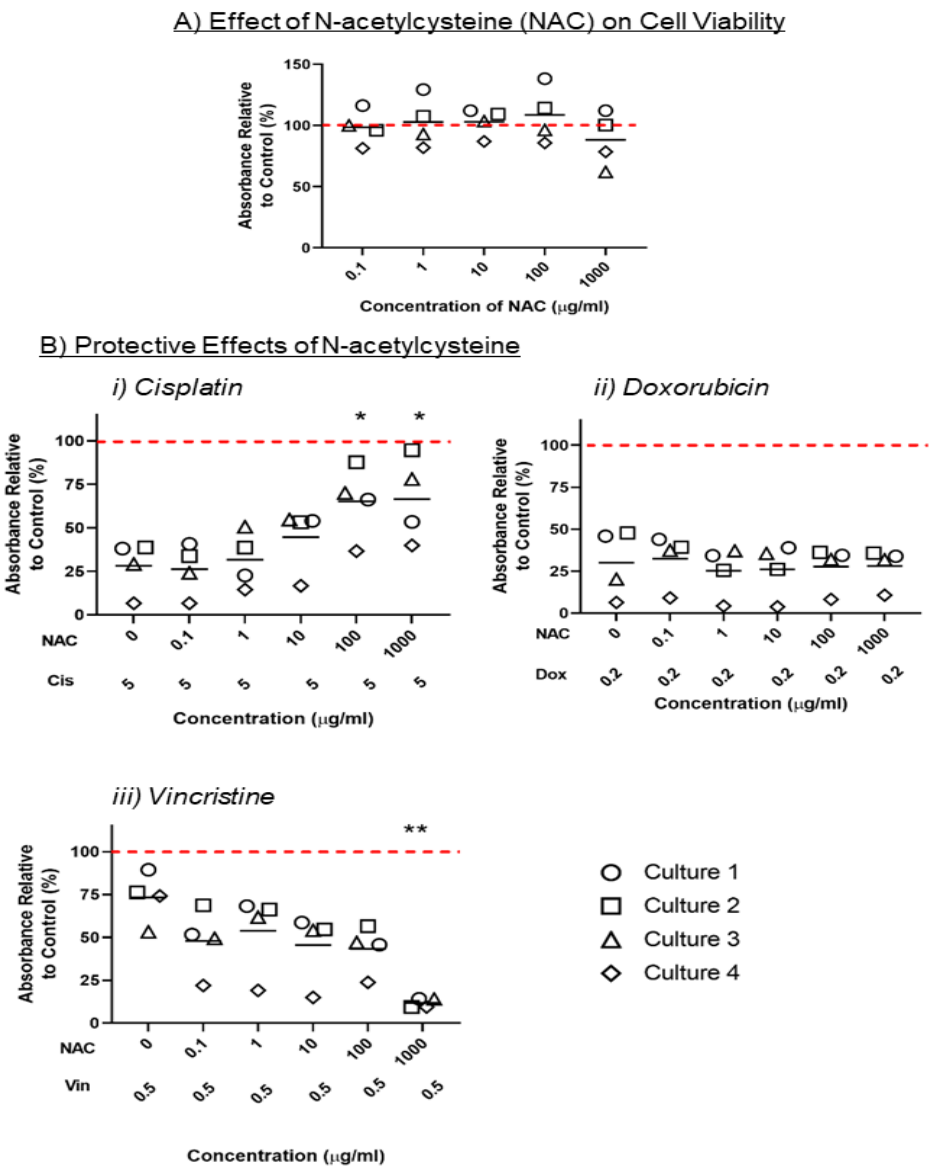


Figure 6.3: Effects of NAC co-treatment on chemotherapy-induced damage to GC-6spg cell line. The impact of NAC (0.1-1000µg/ml) on the GC-6spg cell line**.** B) The protective effects of NAC against i) cisplatin-induced cytotoxicity (5µg/ml), whereas NAC did not ameliorate effects of ii) doxorubicin (0.2µg/ml) cytotoxicity and increased the cytotoxic effects of iii) vincristine (0.5µg/ml). Red line represents the cell viability of control cells at 100%. See Appendix C Figure 6 for QQ-plots analysing normal distribution. A) One-sample t test to compare mean cell viabilities of NAC-exposed cells to 100% cell viability. B) One-way ANOVA and Dunnett's multiple comparisons post-hoc test to compare cell viabilities of co-treated cells to cisplatin-exposed cells. Kruskall Wallis and Dunn's multiple comparisons post-hoc test to compare cell viabilities of co-treated cells to doxorubicin- or vincristine-exposed cells.*p<0.05, **p<0.01. 'n'=4 with data shown in relation to individual culture runs with grand mean.

6.3.3 Preliminary data indicates lycopene not only does not protect against cisplatin, doxorubicin or vincristine-induced damage to the GC-6spg cell line, but is cytotoxic

The protective effects of lycopene co-treatment with cisplatin, doxorubicin or vincristine on the GC-6spg cell line were analysed following 48hrs of exposure, with cell viability determined by MTT assay. From the preliminary data generated from two cultures, lycopene had a negative effect on cell viability on the GC-6spg cell line; cell viability was reduced compared to the 100% control from 0.0001µg/ml (34% cells viable, 'n'=1) to 10µg/ml (27 +/- 12% cell viability, 'n'=2) (Fig 6.4 A). Co-treatment with cisplatin, doxorubicin or vincristine produced similar results where addition of lycopene to cultures either enhanced cytotoxicity or produced similar levels of damage as chemotherapy treatment alone (Fig 6.4 B). Due to the unforeseen negative effects of lycopene from these two cultures, further cultures were not undertaken.

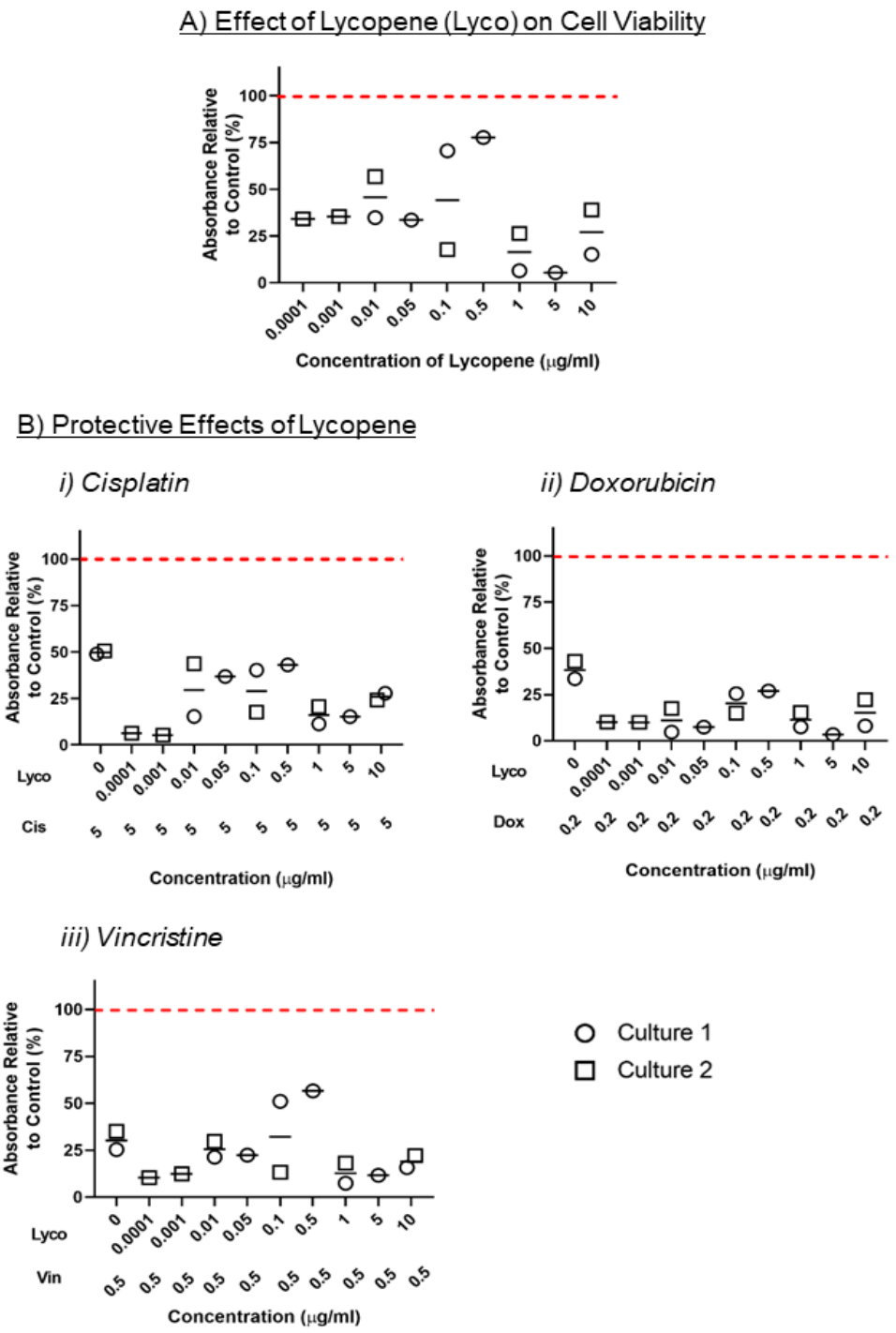


Figure 6.4 Effects of Lycopene co-treatment on chemotherapy-induced damage to GC-6spg cell line. The impact of lycopene (0.0001-10µg/ml) on the GC-6spg cell line**.** B) There was no protective effects of lycopene against i) cisplatin (5µg/ml), ii) doxorubicin (0.2µg/ml) or iii) vincristine (0.5µg/ml) cytotoxicity. Red line represents the cell viability of control cells at 100%. 'n'=2 with data shown in relation to individual culture runs with grand mean.

6.3.4 Preliminary findings of the time-course of apoptosis following co-treatment of GC-6spg cell line with n-acetylcysteine (NAC) and cisplatin or vincristine

To determine the underlying mechanism of action of NAC action upon cisplatin or vincristine co-treatment, the CellEVENT probe was utilized to determine levels of apoptosis following NAC co-treatment with cisplatin or vincristine. Due to Covid-19, these experiments were interrupted and therefore this culture was performed once ('n'=1). The GC-6spg cell line was cultured and co-treated with NAC (0.1, 100 or 1000µg/ml) and cisplatin (5µg/ml) or vincristine (0.5µg/ml) for 48hrs. The CellEVENT probe was also added to culture, with fluorescence (activation of CC3/7) assessed 24 and 48hrs post-exposure, and an MTT assay performed at the end of the experiment to confirm cytotoxicity. NAC concentrations chosen to investigate include a lower dose of 0.1µg/ml that had no protective/synergistic effects, and higher doses of 100 and 1000µg/ml where an effect with cisplatin or vincristine treatment was observed (see 6.3.2). Due to 'n'=1, trends in data were assessed by comparing the averages. Based on this single culture run, NAC treatment alone did not have a substantial effect on levels of apoptosis at either the 24 or 48hr time point, however reductions in cell viabilities were noted (Fig 6.5 i). Levels of apoptosis in co-treated cells with NAC and cisplatin were similar to cisplatin-exposed cells at both the 24 or 48hr time points, with a trend towards increased apoptosis with NAC co-treatment at the 24hr time point and with 0.1µg/ml NAC co-treatment at the 48hr time point (Fig 6.5 Aii, Bii). Indeed, upon measurement of cell viability of this culture run at the 48hr time-point, there were no indications of protection with 100 and 1000µg/ml NAC that had previously been observed (Fig 6.3 Bi), with reduced cell viabilities compared to the cisplatin treated cells (Fig 6.3 Cii). Upon NAC co-treatment of vincristine-exposed cells, increased apoptosis was observed at both time-points compared to the controls and vincristine-exposed cells, with concomitant decrease in cell viabilities compared to vincristine-exposed cells (Fig 6.5 iii). Further cultures are required before any definitive conclusions in regards to NAC's mechanism of action following co-treatment with cisplatin or vincristine can be produced.

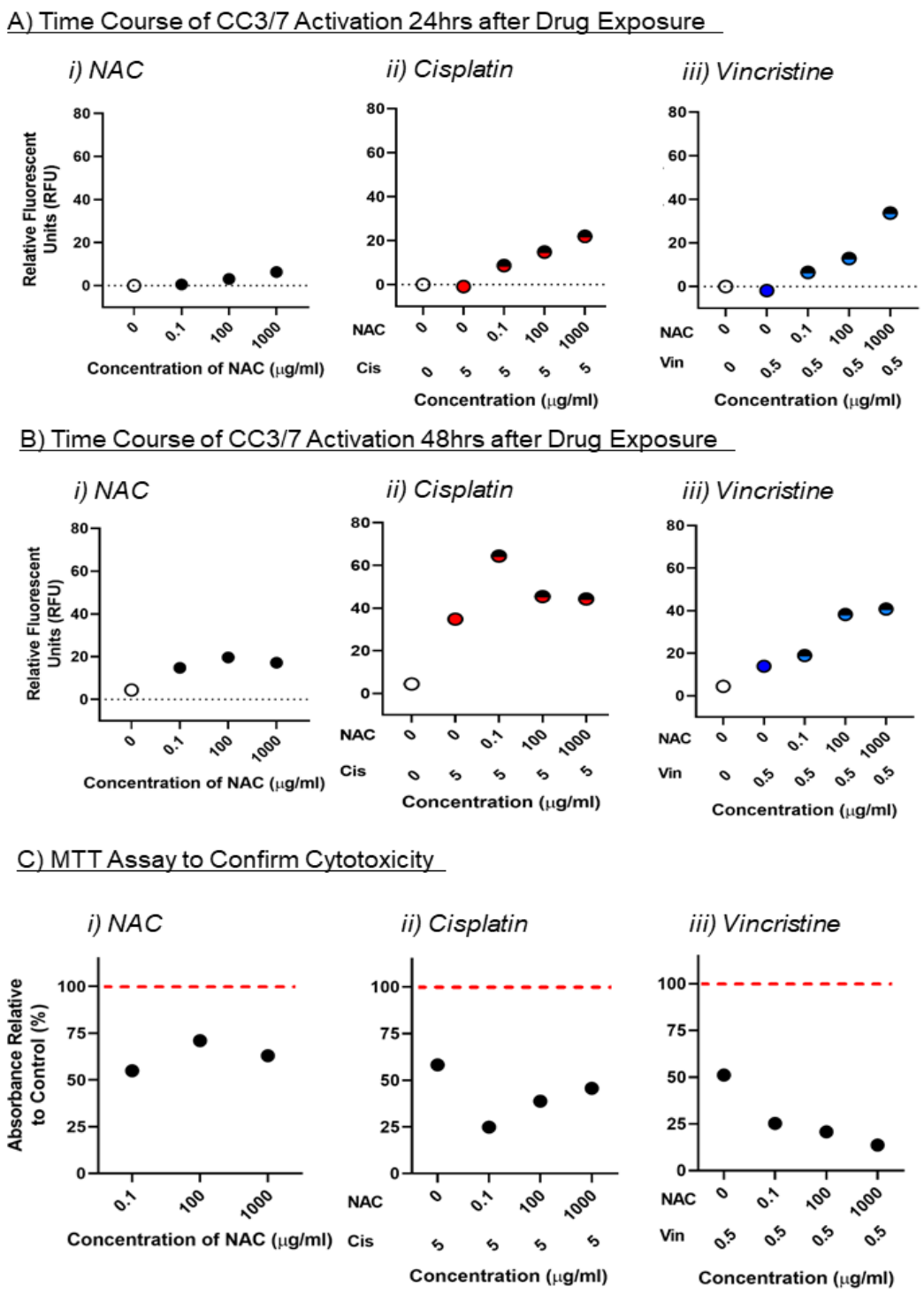


Figure 6.5 Time-course of apoptosis following NAC co-treatment with cisplatin or vincristine with use of CellEVENT probe. Activation of CC3/7 at the (A) 24hr or (B) 48hr time points upon treatment with i) NAC (0.1, 100 and 1000μg/ml), ii) NAC co-treatment cisplatin (5μg/ml) or iii) NAC co-treatment with vincristine (0.5μg/ml). C) Cell viability confirmed at 48hr time point with MTT assay. Red line represents controls cells at 100% cell viability, 'n'=1. Due to low 'n' number, differences between data points were compared by assessing overlap of averages.

6.3.5 Initial observations from culture of prepubertal testis co-treated with n-acetylcysteine (NAC) and cisplatin or vincristine.

To confirm the findings from NAC co-treatment with the GC-6spg cell line, further cultures were undertaken with co-exposure of mouse prepubertal testicular tissues for 24hrs to NAC (5 and 100µg/ml) and cisplatin (0.5µg/ml) or vincristine (0.1µg/ml). The density of the germ cells, including proliferating and non-proliferating, were assessed per area of section with immunofluorescence at the end of the four-day culture period. NAC concentrations investigated include 5µg/ml NAC, a concentration observed in human serum after supplementation (serum levels 0.35-20.8µg/ml; Holdiness, 1991; Pendyala and Creaven, 1995), as well as 100µg/ml NAC at which protective/synergistic effects were observed in the above GC-6spg cell line experiments (6.3.2). Dosages of chemotherapy agents were selected based on the dose-response experiment (see Chapter 4.3.1 and 5.3.1); with concentrations that induced a significant decrease in germ cell densities chosen that were not exceedingly toxic to the tissues i.e. did not kill off all germ cells present. Based on this preliminary culture run, there was no obvious change in MVH expression levels (green signal) in the immunofluorescent images of NAC treated tissues, where as a decrease with cisplatin or vincristine treatment was seen, compared to the control (Fig 6.6). This indicated NAC did not affect the density of the germ cell population. These initial observations were confirmed upon manual counting of germ cells, proliferating and non-proliferating, with similar densities for NAC exposed tissues compared to control, with reductions in cisplatin or vincristine-exposed tissues in comparison to the control tissue (Fig 6.7). With vincristine treatment alone, fewer germ cells were apparent in this tissue, however the localisation of germ cells in the centre of seminiferous tubules (as noted in Chapter 4, see 4.3.1) was not observed at the concentration tested. Upon NAC co-treatment with cisplatin, there was no apparent difference in the expression levels of MVH (green), confirmed with manual counting of germ cells, compared to the cisplatin-exposed tissues (Fig 6.6 iv-vi, 6.7 ii). Remarkably, NAC co-treatment with vincristine results in localisation of the germ cells into the centre of seminiferous tubules, which was not observed with vincristine

treatment alone (Fig 6.6 vii-ix). Reductions in the number of germ cells was apparent, although similar compared to the vincristine treated tissues (Fig 6.7 iii). Four culture runs were set-up for these experiments, however due to Covid-19 only one run was analysed and therefore additional laboratory-based work is required before overall conclusions can be drawn from the data.

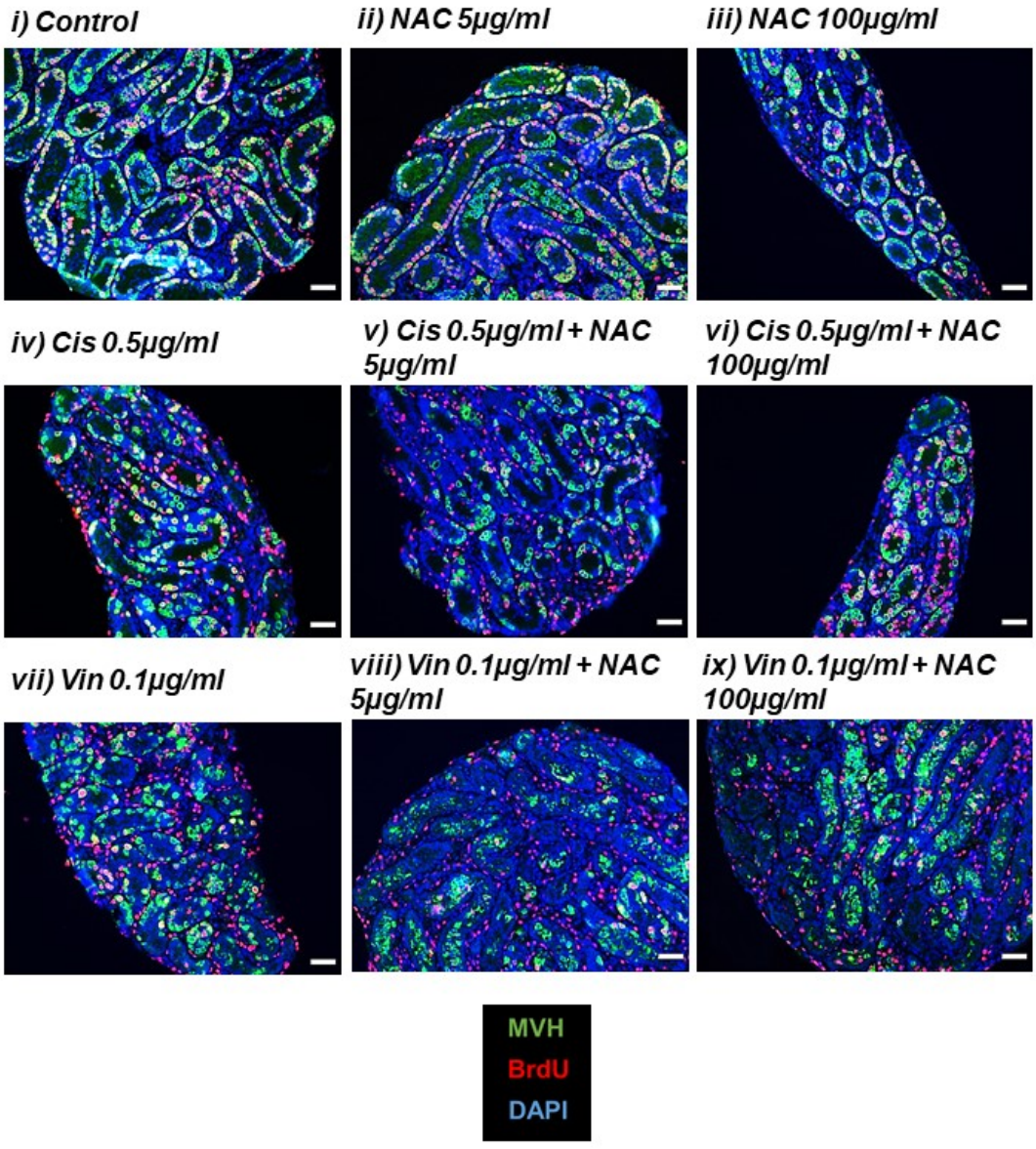


Figure 6.6 Impact of NAC co-treatment with cisplatin or vincristine on prepubertal testis. Representative images of i) control, ii) NAC 5µg/ml, iii) NAC 100µg/ml, iv) cisplatin (0.5µg/ml), v) NAC 5µg/ml co-treatment with cisplatin, vi) NAC 100µg/ml co-treatment with cisplatin, vii) vincristine (0.1µg/ml), viii) NAC 5µg/ml co-treatment with vincristine or ix) NAC 100µg/ml co-treatment with vincristine.

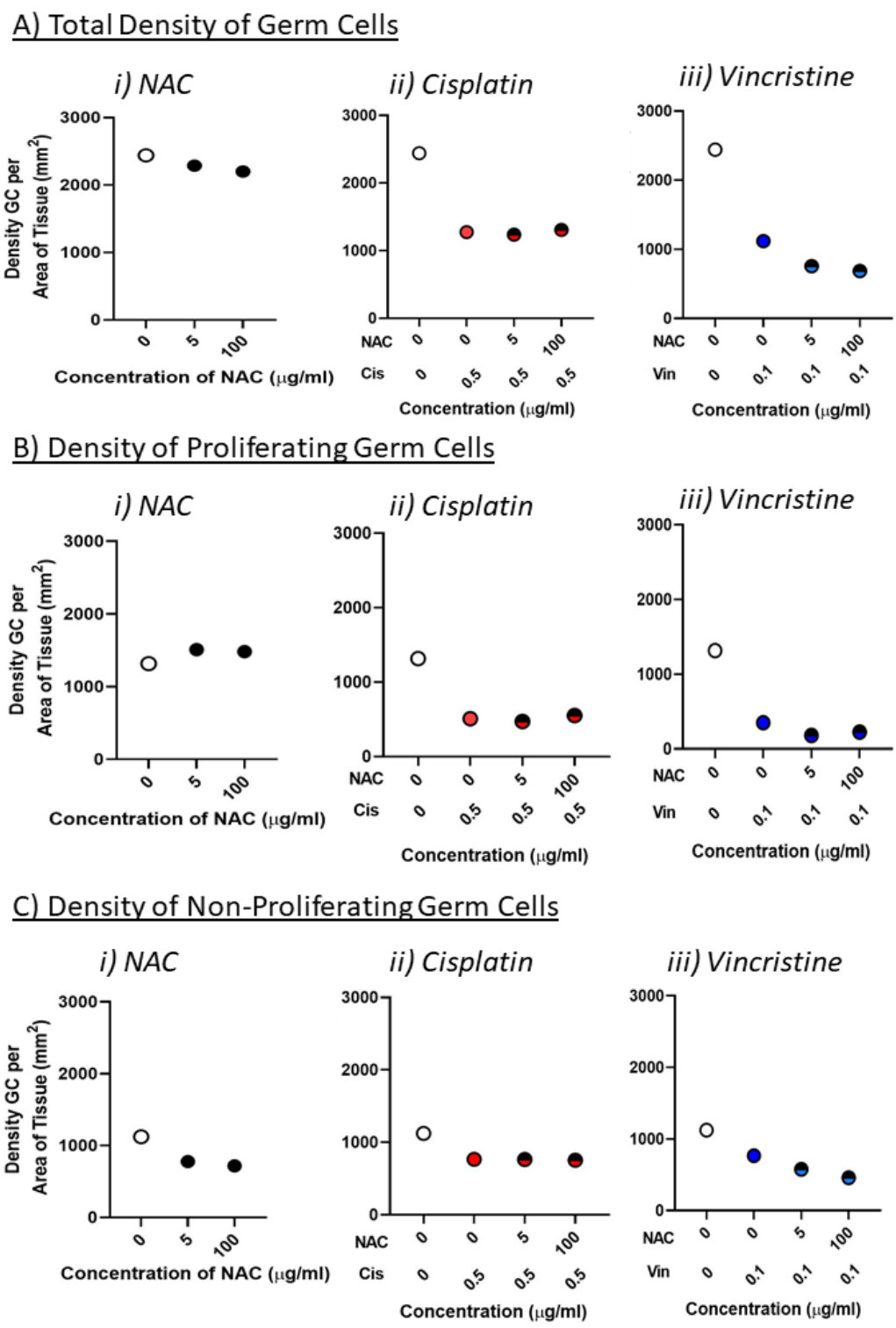


Figure 6.7 Analysis from a culture run with co-treatment of prepubertal testis with NAC and cisplatin or vincristine. A) Density of total germ cell population, B) proliferating and C) non-proliferating germ cells per area of tissue following i) NAC (5 and 100µg/ml) treatment, ii) NAC co-treatment with cisplatin (0.5µg/ml) or iii) NAC co-treatment with vincristine (0.1µg/ml).

6.4 Discussion

6.4.1 Overview of results

The inclusion of antioxidants in cancer treatment regimens has the potential to mitigate the negative side effects associated with chemotherapy treatments on healthy tissues such as the reproductive tissues. Within this chapter, the protective capacity of the antioxidants melatonin, NAC and lycopene against cisplatin-, doxorubicin- or vincristine-induced damage was investigated primarily in the rat GC-6spg cell line and also the mouse prepubertal testis. Cell viability of GC-6spg cell line and density of germ cells in testis fragments were assessed following co-treatment of antioxidants with chemotherapy agents for 48hrs or 24hrs, respectively. High concentrations of melatonin partially protected the GC-6spg cell line against cisplatin, whereas no protective effects were observed upon co-treatment with doxorubicin or vincristine. Unexpected results were obtained following NAC co-treatment since NAC partially protected against cisplatin, enhanced cytotoxic effects of vincristine and had no impact on doxorubicin-induced damage to the GC-6spg cell line. Initial cultures were also undertaken to determine the underlying mechanism of action of NAC effects and whether they translated to tissue with culture and co-treatment of prepubertal testis fragments. Lycopene did not protect against chemotherapy treatment and itself was cytotoxic to the GC-6spg cell line. The results from this chapter highlight the potential protective effects and, remarkably, the negative effects that antioxidants such as melatonin and NAC have upon the SSC-like GC-6spg cell line following co-treatment with chemotherapy agents.

6.4.2 Effects of melatonin co-treatment on chemotherapy-induced cytotoxicity

Melatonin is an endogenous, non-enzymatic antioxidant that is produced from the pineal gland in the body and can be given as a supplement to aid sleeping (reviewed in Tordjman *et al.*, 2017). Within this study, the protective effects against a cytotoxic dose of cisplatin (5µg/ml) was apparent following culture and co-treatment of the GC-6spg cell with a high dose of 20µg/ml melatonin for 48hrs. The protective capacity of melatonin in the adult male reproductive system has been investigated in a

number of *in vivo* animal model studies against cisplatin-induced damage alone (Ateşşahin *et al.*, 2006a; Ilbey *et al.*, 2009; El-shafaei *et al.*, 2018; Eren *et al.*, 2020) or in combination with bleomycin and vinblastine (Madhu *et al.*, 2016). Within these studies, the role of oxidative stress in cisplatin-induced damage to the adult testis is clear with increased levels of MDA (oxidative stress marker) as well as decreased levels/activity of glutathione, SOD and glutathione peroxidases (antioxidants). Cisplatin treatment (~7mg/kg) produced severe degeneration of the testis with Sertoli-cell only tubules/reductions in numbers of germ cells, reduced germinal tubule thickness and interstitial oedema. Melatonin treatment with different regimens, including daily injections of 5-20mg/kg for five days before or up to ten days after cisplatin treatment, partially protected the testis from cisplatin-induced damage. The improvement in the histology is considered to be due to the antioxidant activity of melatonin since levels of oxidative stress were reduced and the antioxidant capacity of the testis increased upon melatonin treatment. These studies focused mainly upon the sperm morphology, motility etc. with histology assessed from H&E stained sections and scoring of tissues with Johnsens seminiferous tubular damage score (Ateşşahin *et al.*, 2006a; Ilbey *et al.*, 2009; El-shafaei *et al.*, 2018; Eren *et al.*, 2020). In the Eren *et al.* (2020) study, there is an obvious loss of spermatocytes, spermatids and spermatozoa with abnormal Sertoli cells and pyknotic spermatogonia and Leydig cells following cisplatin treatment. In addition, the study scored images from immunohistochemistry for CC3-positive (apoptotic) and 8-OhdG-positive (DNA damage oxidative stress) cells within the tubules (expression restricted to later stages of spermatogenesis) and interstitium, which were increased upon cisplatin treatment. Melatonin treatment of 5mg/kg for five days before cisplatin exposure was able to partially ameliorate the negative effects of cisplatin. The findings from this thesis confirm a protective effect on the SSC/spermatogonia GC-6spg cell line with 48hrs of co-exposure of the cells to melatonin. The dose of 20µg/ml melatonin, however, is 5000X higher than the highest serum levels of melatonin noted in humans (400—4000pg/ml) and therefore is not feasible as a therapeutic dose and was initially included in this thesis to determine a dose-response. Experiments with

longer exposure periods with lower doses of melatonin should therefore be undertaken either *in vitro* or *in vivo* to determine whether there is potential for these protective effects to be relevant and worth further investigation.

Melatonin co-treatment with doxorubicin or vincristine did not protect the GC-6spg cell against cytotoxicity as measured through cell viability in this study. This confirms the hypothesis in relation to vincristine cytotoxicity since this agent is not considered to induce oxidative stress as an underlying mechanism of action (reviewed in Conklin, 2004). However, the hypothesis in regards to doxorubicin indicated that melatonin would protect against damage since this agent is known to induce oxidative stress as a mechanism of action, indeed this action is associated with known toxicities of doxorubicin such as cardiotoxicity (reviewed in Conklin, 2004). Evidence of melatonin protection against doxorubicin-induced damage to the reproductive system is scarce with to date only one study investigating the protective effects of melatonin in the adult reproductive system of Sprague dawley rats (Lee *et al.*, 2012). In this study, there was loss of the spermatogonia and early spermatocytes associated with doxorubicin treatment, with melatonin treatment partially protecting these germ cells. This protection was attributed to alleviation of oxidative stress (MDA levels) and improvement in antioxidant capacity (glutathione content, glutathione enzymes, SOD and CAT) of the tissue. The GC-6spg cell line is representative of the early germ cells, the SSCs and spermatogonia, which are present in the prepubertal testis and therefore adds weight to our initial hypothesis. The lack of a protective effect in this thesis may be due to the exposure length, since in the Lee *et al.*, (2012) study the rats were treated with melatonin (15mg/kg) for five days before doxorubicin treatment (10mg/kg) and five days after. Further experiments therefore should focus on extending the exposure period of melatonin, as discussed above, before ruling out melatonin as a protective agent against chemotherapy-induced to the prepubertal testis.

6.4.3 Effects of n-acetylcysteine (NAC) co-treatment on chemotherapy-induced cytotoxicity

NAC has known antioxidant properties and increases the production of glutathione (reduced GSH), this drug is commonly administered for overdose of paracetamol (reviewed in Heard, 2008). The protective capacity of NAC was initially investigated upon co-treatment of NAC (0.1-1000µg/ml) with cytotoxic doses of cisplatin, doxorubicin or vincristine in the GC-6spg cell line for 48hrs. In regards to the GC-6spg cell line, whether NAC has an effect is of interest since an earlier study found low levels of glutathione in this cell line, which may be biological or due to monoculture conditions (Tremblay and Delbes, 2018). NAC co-treatment, remarkably, produced differential effects with each drug whereby high concentrations partially protected against cisplatin cytotoxicity, whilst enhancing cytotoxic effects of vincristine and having no impact on doxorubicin toxicity.

The protective effects of NAC against cisplatin-induced toxicity to the male reproductive system has been investigated to date in one animal model study which used NAC as a known antioxidant to compare to the protective effects of a herbal extract, *Eugenia jambolana* (Anand *et al.*, 2015). In this study, NAC (150mg/kg) administration on day of cisplatin dosage (5mg/kg) and three days later was able to prevent cisplatin-induced oxidative stress (MDA levels) and augmented antioxidant capacity (reduction in level/activity of glutathione, glutathione enzymes, SOD and CAT activity) of the tissue. This relative low dose of cisplatin did not induce significant morphological changes to the tissue; however, at a molecular level NAC treatment was able to attenuate the number of TUNEL positive cells and activation of intrinsic and extrinsic apoptosis following cisplatin treatment in the testis. This was considered to be due to the antioxidant activity of NAC. On a hormonal level, NAC co-treatment did not protect against increased FSH and reduced LH and testosterone levels associated with cisplatin treatment, however, these changes may be associated with damage to HPG axis. To investigate the underlying mechanism of action of NAC protection with the GC-6spg cell line, the CellEVENT probe, which

fluoresces upon activation of CC3/7, was included with co-treatment of cytotoxic dose of cisplatin with 0.1, 100 and 1000µg/ml NAC. Due to Covid-19 however, only one run of this culture was undertaken and the protective effects previously shown were not observed. Further cultures should therefore be undertaken with this CellEVENT probe and, upon additional optimization, the CellROX probe to assess for ROS production to determine the molecular and mechanistic protection of NAC against cisplatin-induced cytotoxicity. It has been suggested in Conklin and Conklin, (2000) (based on the study (Appenroth *et al.*, 1993)) that since NAC is a nucleophile substance it may bind to the electrophile platinum-containing cisplatin and effectively inactivate the drug. This inactivation would decrease the efficiency of cisplatin as an anti-cancer agent and may account for the observation in Anand *et al.* (2015) and those within this thesis since cells were co-treated with NAC and cisplatin. Indeed, in Muldoon *et al.* (2015) study, the timing of NAC administration was very important when determining the effectiveness of NAC to protect against cisplatin-induced nephrotoxicity in a rat paediatric tumour model. Administration of NAC before cisplatin treatment reduced the effectiveness of this anti-cancer agent, potentially by binding to and inactivating the drug, whilst delaying NAC treatment for 4hrs maintained the anti-cancer action of cisplatin as well as providing protection against chemotherapy-induced nephrotoxicity. Further cultures focusing upon NAC as a chemo-protectant against cisplatin should therefore consider the timing of NAC treatment, for example exposing prepubertal testis fragments to NAC for the final two-days of culture. Initial cultures have been performed to determine whether the protective effects observed in the GC-6spg cell line translate to cultured prepubertal testis tissue following 24hrs of co-treatment. Based on a single culture run due to Covid-19, however, there is no indication that NAC protected the germ cell population in the prepubertal testis from cisplatin-induced gonadotoxicity upon co-treatment.

Doxorubicin-induced cytotoxicity to the GC-6spg cell line was not attenuated upon co-treatment with NAC for 48hrs. As discussed in relation to melatonin, the original

hypothesis stated that protective effects would be observed with use of NAC since doxorubicin is known to induce oxidative stress as a mechanism of action (reviewed in Conklin, 2004). In addition to the antioxidants investigated in this thesis, the protective effects of vitamin C, curcumin, amifostine and cartinine have been tested against doxorubicin-induced cytotoxicity in the GC-6spg cell line and Ser-W3 cell line (immature Sertoli cell) by Tremblay and Delbes, (2018). This study also found no indication of protective effects. Based on this evidence, they concluded oxidative stress may not be heavily involved as an underlying mechanism of toxicity following doxorubicin treatment. In addition, due to the low levels of glutathione in the GC-6spg cell line, they also proposed that the glutathione enzymes are not heavily involved in protecting this cell line against oxidative stress. Our data is in agreement with these general conclusions since no protective effects were noted for melatonin or NAC co-treatment. This, however, may be a specific culture/cell line effect as there have been protective effects of antioxidant compounds against doxorubicin-induced gonadotoxicity noted from *in vivo* prepubertal animal model studies (see 1.7.2.1 and 1.7.2.2) (Jahnukainen *et al.*, 2001; Hou *et al.*, 2005; Vendramini *et al.*, 2010, 2012, Cabral *et al.*, 2014, 2018; Abdelaziz *et al.*, 2019). There are no studies, to this author's knowledge, investigating the protective effects of NAC against doxorubicin-induced toxicity to the adult reproductive system to enable comparison. Future investigations may therefore want to consider using a tissue culture or *in vivo* animal model study to determine protective effects of antioxidants against doxorubicin-induced toxicity.

NAC co-treatment with vincristine produced unexpected results whereby NAC increased the cytotoxicity of vincristine against the GC-6spg cell line, reducing cell viability to an extent which was rarely observed with vincristine treatment alone. Upon further analysis of the underlying mechanism of action, increased levels of CC3/7 expression following co-treatment with 100 or 1000µg/ml of NAC suggests NAC co-treatment enhances apoptosis within this cell line. The synergistic effects of NAC and vincristine were also confirmed with the prepubertal testis culture model whereby co-treatment of NAC at 5 and 100µg/ml with vincristine enhanced the

accumulation of the germ cells into the centre of the seminiferous tubules. These results were unexpected, since NAC was hypothesised to have no effect upon vincristine-induced damage to the GC-6spg cell line/prepubertal testis since this agent is not considered to induce oxidative stress as a mechanism of action (reviewed in Conklin, 2004). These results were initially puzzling to say the least, but following discussion a hypothesis was formed that NAC interferes with stress–induced expression of p-glycoprotein that leads to increased intracellular accumulation of vincristine, resulting in increased cytotoxicity (personal communication between supervisor N Spears and Dr Beth Coyle, University of Nottingham). P-glycoprotein is a membrane bound, ATP-dependent, transporter protein that acts as an efflux pump to remove drugs from the cell, thereby reducing intracellular concentrations and efficacy of the agent (reviewed in Goldman, 2002). These p-glycoproteins have been studied extensively for their role in drug resistance in tumour cells and are commonly known as multidrug resistance proteins 1 (MDR-1), which are encoded by the multi-drug resistance gene (reviewed in Silva *et al.*, 2015). Vincristine, as well as doxorubicin and cisplatin, are inducers of p-glycoprotein and therefore increase the expression of these transmembrane proteins. These drugs are also substrates of p-glycoproteins and are exported from the cells through these efflux pumps, conferring resistance to these chemotherapy agents (Chaudhary and Roninson, 1993; Harmsen *et al.*, 2010). P-glycoproteins are found in the testis with expression noted in the somatic cell populations; however, p-glycoproteins have been proposed as a marker of stem cells and could therefore be expressed in the SSC population (reviewed in Bunting, 2002). Indeed, the GC-6spg cell line is SSC representative and could therefore express p-glycoprotein. NAC has been shown to interfere with oxidative-stress induced expression of p-glycoprotein; this would result in reduced numbers of these efflux pumps at the plasma membrane and would therefore lead to accumulation of drugs within the cell (Zhang *et al.*, 2017). Oxidative stress is not involved in the toxicity of vincristine; however, free radicals and therefore oxidative stress can be induced upon activation of cell death pathways that could signal stress-induced p-glycoprotein expression. This would therefore account for increased

vincristine cytotoxicity upon NAC co-treatment whereby NAC prevents the oxidative-stress induced accumulation of p-glycoprotein in the GC-6spg cell/prepubertal testis. This synergistic NAC effect, however, was not observed for the other chemotherapy drugs investigated in this thesis that are considered known inducers of oxidative stress. Further investigations are required to test this working hypothesis to determine the expression of p-glycoproteins in the GC-6spg cell line and SSCs in the prepubertal testis and the effects chemotherapy treatment and NAC has upon these transporters. Other mechanisms of NAC action could also be responsible for the synergistic effects of vincristine. In addition to acting as a direct ROS scavenger, NAC increases glutathione concentrations which has been shown to be involved in the protection of a human embryonic kidney cell line against vincristine induced cytotoxicity (Akan *et al.*, 2005). These results, however, are opposite to the effects of NAC and vincristine co-treatment observed in this thesis, highlighting the need for further research.

6.4.4 Effects of lycopene co-treatment on chemotherapy-induced cytotoxicity

The protective effects of lycopene were tested following co-treatment of 0.0001-10µg/ml lycopene with cytotoxic doses of cisplatin, doxorubicin or vincristine in the GC-6spg cell line. Lycopene, itself, however was found to be cytotoxic to the GC-6sgp cell line with cell viability reduced by less than 50% compared to the controls. This cytotoxic effect was noted from the lowest dose of 0.0001µg/ml, which is well below plasma supplementation levels of lycopene noted after tomato product consumption (0.07-1.04µg/ml) (Jenab *et al.*, 2005; Datta *et al.*, 2013). The protective effect of lycopene against cisplatin and doxorubicin has been shown with *in vivo* animal model studies, to an extent (Ateşşahin *et al.*, 2006b, c; Türk *et al.*, 2011; Salem *et al.*, 2012). In a short-term study, the protective effect of lycopene (4mg/kg) pre-treatment for ten days before cisplatin (7mg/kg) treatment or five days post cisplatin treatment were shown in relation to sperm parameters, however the thickness of the germinal epithelium was not protected against cisplatin damage (Ateşşahin *et al.*, 2006b). Chronic treatment of cisplatin (2mg/kg per week) and lycopene (10mg/kg every other

day) did protect the testis against cisplatin-induced damage with improvements in histology and reductions in the number of apoptotic germ cells. This was associated with attenuating cisplatin-induced lipid peroxidation. In regards to doxorubicin, the protective effect of ten days of lycopene (4mg/kg) pre-treatment against a gonadotoxic dose of doxorubicin (10mg/kg) was apparent with improvements in testis morphology linked to reductions in lipid peroxidation (Ateşşahin *et al.*, 2006c). The negative effects of lycopene on the GC-6spg cell line could therefore be a culture effect or specific to the cell line. Further investigations could take place with cultured prepubertal testis tissue or *in vivo* animal model work to determine whether lycopene can protect against cisplatin or doxorubicin-induced gonadotoxicity to the prepubertal testis, as observed in relation to the adult testis.

6.4.6 Conclusions

To conclude the findings from this chapter, melatonin and NAC partially protected against cisplatin-induced damage to the GC-6spg cell line at high doses, whereas NAC co-treatment with vincristine enhanced the cytotoxic effect of this drug. Of the antioxidants investigated, none were able to attenuate doxorubicin-induced cytotoxicity, while the antioxidant lycopene was itself cytotoxic to the GC-6spg cell line. Work here produced a number of unexpected results, particularly with the negative effects of NAC co-treatment with vincristine and lycopene treatment of the GC-6spg cell line. Initial cultures were undertaken to determine the underlying mechanism of action of vincristine and NAC synergistic effect, which indicate potential enhanced apoptosis upon co-treatment. These findings with the GC-6spg cell line were also confirmed in the prepubertal testis culture model with co-treatment of NAC and vincristine. The results from this chapter are the initial investigations into the protective effects of antioxidants against chemotherapy-induced damage to the prepubertal testis utilizing the mouse prepubertal testis culture model and a cell line representative of SSCs, rat GC-6spg, which are found within the prepubertal testis. Further investigations, for example using other markers of oxidative stress, are required to confirm the underlying mechanism of protection

of melatonin and NAC against cisplatin-induced damage to determine whether these agents reduce oxidative stress as the molecular mechanism of protection, or perhaps through other known actions of these agents. Optimization of the concentration and treatment regimens of these antioxidants is also required since protection was observed at high concentrations. There are a wide range of antioxidants, including selenium, vitamin E and coenzyme Q10 that could be investigated with use of these culture model systems that allow rapid initial investigations to determine the potential of these agents.

Chapter 7. Discussion

7.1 Summary of Results

The aim of this PhD was to examine the gonadotoxicity and underlying mechanisms of action of chemotherapy drugs used to treat childhood cancer, as well as the potential of antioxidants to protect the prepubertal testis against chemotherapy-induced damage as a fertility preservation strategy. This involved exposing cultured prepubertal mouse testis fragments and the rat GC-6spg cell line (representative of SSCs) to chemotherapy agents and/or antioxidants, and determining gonadotoxicity and levels of oxidative stress as an underlying mechanism of action.

Newer generations of chemotherapy agents are often developed and introduced to clinical practise in order to reduce the dose-limiting side effects of treatment. Indeed, in certain instances the second-generation drug carboplatin can be used to replace cisplatin, the first-generation drug within the platinum alkylating-like class. Whether carboplatin is less gonadotoxic compared to cisplatin was unknown, and hence investigated here by culturing prepubertal mouse gonads (Chapter 3). Results suggest that carboplatin could be no less gonadotoxic than cisplatin at clinically relevant doses in a mouse *in vitro* model.

Production of ROS and oxidative stress were investigated as the potential underlying mechanism of action of cisplatin-, doxorubicin- or vincristine-induced gonadotoxicity (Chapter 5). Previous research within our laboratory had shown dose-dependent effects of cisplatin or doxorubicin treatment with use of cultured mouse prepubertal gonads (Morgan *et al.*, 2013; Smart *et al.*, 2018). Evidence also suggested that oxidative stress is involved in the general underlying mechanism of action, although unknown in regards to gonadotoxicity (reviewed in Conklin, 2004). Within this thesis, the role of ROS and oxidative stress in chemotherapy-induced testicular toxicity could not be determined, with further optimization of methods of analysis required (Chapter 5). Vincristine was included in this investigation as a 'negative' control, since

this agent had neither been considered particularly gonadotoxic nor thought to induce oxidative stress as a mechanism of action (reviewed in Conklin, 2004; Wyns *et al.*, 2010). Here, gonadotoxicity was apparent following vincristine treatment in both culture models. Interestingly, in the prepubertal testis large clusters of germ cells were observed within in the centre of the seminiferous tubules, a phenomenon that has not been found following exposure to other chemotherapy agents investigated to date (Chapter 4). Preliminary data show that these germ cells are absent from the testis following a longer culture period, indicating they eventually undergo cell death. Nonetheless, it exists a possibility for seminiferous tubule repopulation, due to the continued presence of a few SSCs along the basement membrane.

Finally, the antioxidants melatonin, NAC and lycopene were investigated as potential cytoprotective agents (Chapter 6). These different antioxidants each produced specific results in the GC-6spg cell line experiments, with melatonin and NAC partially protecting against cisplatin-induced cytotoxicity, whilst in contrast NAC exacerbated vincristine cytotoxicity, with lycopene found to be cytotoxic itself. No protection against doxorubicin-induced cytotoxicity was observed in any instance, questioning the hypothesis that oxidative stress may be involved in its mechanism of action in the testis. Overall, results indicate that antioxidants can potentially have both beneficial and detrimental effects depending upon chemotherapy agents involved.

7.2 Use of Cytoprotectants to Protect Fertility

Protecting the prepubertal testis from chemotherapy-induced damage with the use of cytoprotective agents is a fertility preservation strategy under investigation, but to date has only been successful in animal models. The practicality of using such agents as an adjuvant to chemotherapy treatment to reduce associated side effects has been shown with a number of agents that are FDA approved for inclusion during cancer therapy (reviewed in Hogle, 2007). These include amifostine, which may reduce oxidative stress, to protect against cisplatin-induced nephrotoxicity; dexaroxane to

protect against doxorubicin-induced cardiomyopathy; and mesna to reduce cyclophosphamide-induced haemorrhagic cystitis. To determine whether cytoprotective agents could be used as a fertility preservation strategy to protect against chemotherapy-induced toxicity, a greater understanding of the gonadotoxicity and underlying mechanism of action of chemotherapy agents and potential cytoprotectants is required.

At present, there is little clinical data examining the effects of chemotherapy treatment during childhood, the resulting gonadotoxicity or any later impact on fertility. Associations between use of alkylating and alkylating-like agents, and infertility in adulthood has been shown in clinical data either through morphological examination of the testis or assessment of reproductive hormone levels during treatment and/or in adulthood (reviewed in Kelsey *et al.*, 2017; Skinner *et al.*, 2017; Tian En *et al.*, 2020). However, due to the use of multiple chemotherapy agents of different classes in treatment regimens, it has been hard to define the relative contributions of different drugs. Results from this thesis, based on *in vitro* animal model data, suggests that some chemotherapy agents that are currently considered a low risk agent for later infertility, such as vincristine, are potentially gonadotoxic to the prepubertal testis, at least in the short-term (Chapter 4). In addition, newer generations of drugs that reduce some dose-limiting side effects may not translate to a reduction in gonadotoxicity, as suggested in the case of carboplatin compared to cisplatin here (Chapter 3). Further research is required to provide evidence for the current gonadotoxicity classifications of chemotherapy agents for prepubertal patients. This is vital, not only for research into cytoprotective agents, but also to enable clinicians to provide patients with more information about how treatments could potentially affect subsequent fertility. Due to the scarcity of human prepubertal testis tissue available for research, the use of animal models are an important tool for reproductive toxicity testing (see below for more details 7.3).

Determining the underlying mechanism of toxicity of chemotherapy agents is key to aid development of cytoprotective agents; this was the approach taken during this PhD to understand the role of oxidative stress and therefore the potential of antioxidants to prevent chemotherapy-induced toxicity. From my point of view, the molecular targets and underlying mechanism of action of chemotherapy agents are not well understood. Indeed this is why I first became interested in oxidative stress upon researching cisplatin and doxorubicin. Cisplatin intercalates into DNA resulting in double-strand breaks and DNA damage, as well as inducing oxidative stress (reviewed in Dasari and Tchounwou, 2014). Doxorubicin is a topoisomerase II inhibitor that intercalates into DNA resulting in DNA damage, and also produces ROS (reviewed in Thorn *et al.*, 2011). This raised the question; what are the relative contributions of these underlying mechanisms of action to gonadotoxicity? That question led to the research undertaken during this PhD. Previous research within our laboratory had assessed DNA damage following cisplatin or doxorubicin exposure by examining γH2AX, an early response to DNA double strand breaks whereby the H2AX histone variant is phosphorylated (Smart *et al.*, 2018). Expression of γH2AX increased 16hrs following treatment, with further research required to determine the contribution of DNA damage to gonadotoxicity. This could take place with use of inhibitors of DNA damage and/or phosphorylation for H2AX, such as inhibitors of ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PKcs (DNA-dependent protein kinase), which are kinases activated upon DNA damage (reviewed in Maréchal and Zou, 2013). Since oxidative stress can induce DNA strand breaks resulting in activation of these pathways, as shown in human spermatozoa (Li *et al.*, 2006), it would be difficult to determine the contributions of each event in the molecular toxicological process and, most importantly, the cause-effect relationship.

Antioxidants have been investigated as cytoprotective agents for nearly two decades, however many questions remain as to the efficacy and safety of inclusion in cancer therapy. Upon identification of agents that potentially protect against chemotherapy-induced damage to healthy tissues/cells, determining whether these

agents disrupt the anti-cancer activity upon malignant tumours is essential. Such investigations could be performed with use of paediatric tumour samples or cell lines exposed to the antioxidants and chemotherapy agents (see Harada *et al.* (2002) for examples of paediatric cell lines). Paediatric cancer cell lines could also be injected into nude mice to form tumours, with assessment of tumour growth following chemotherapy and antioxidant treatment. These investigations would provide preliminary data as to whether antioxidants interfere with cancer progression and/or effectiveness of chemotherapy treatments, before preclinical studies are performed. Indeed, an ideal situation would involve using a cytoprotective agent that also has anticancer activity, as proposed for melatonin (reviewed in Di Bella *et al.*, 2013). Ultimately, the use of antioxidants as an adjuvant to chemotherapy treatment will most likely be patient specific and require a fine balance, with a mixture of antioxidants potentially more beneficial than treatment with individual antioxidants (reviewed in Singh *et al.*, 2018). This however, would require a substantial amount of research, since as shown in this thesis; an antioxidant that protects against one chemotherapy agent may be ineffective or even exacerbate the effects of another.

Understanding the molecular targets and mechanistic pathways involved in cytoprotectant activity is crucial before acceptance of such agents for clinical use. There are, for example, multiple herbal extracts that contain antioxidant activities that could theoretically be used as cytoprotective agents; however, many clinicians/scientists are wary of their use due to the unknown constituents, molecular targets and mechanisms of action (reviewed in Fu *et al.*, 2018). In addition, there is no evidence that such agents reduce gonadotoxicity in clinical practise. It is of importance to determine whether the cytoprotectants protect the testis against chemotherapy-induced damage or merely prevent any damaged germ cells from undergoing cell death, which would in effect result in the maintenance of unhealthy germ cells (see for example Vendramini *et al.*, 2012). Such investigations could include immunohistochemistry to assess for damaged germ cells in the short-term, for example expression of cleaved caspases or DNA/RNA damage (γH2AX) following

cytoprotectant treatment. In addition, long-term *in vivo* studies to assess the well-being of the next generation would provide information on the health of maintained germ cells.

Delivering cytoprotective agents to the testis without having systematic or local interactions, as in the case of oral or intravenous administrations, would be an ideal solution to prevent/limit the cytoprotective agents from interfering with anticancer activity and the cancer, and to deliver high therapeutic doses. The testis itself is isolated from the body; therefore, intratesticular injections are a possible route for drug delivery directly to the targeted tissue, as has been shown in a rodent model (Russell *et al.*, 1987). This could involve injection directly into the rete testis or parenchyma; however, systematic effects are still possible due to transmission of cytoprotective agents to the rest of the body through the testis vasculature. Cytoprotective agents could therefore interfere with the cancer and anti-cancer treatment, perhaps particularly for patients with acute lymphoblastic leukaemia since the testis is known to harbour leukaemia infiltrates (Kim *et al.*, 1986). Cytoprotectant delivery may be a time-consuming and invasive procedure, depending upon the route of administration and frequency of treatments needed to limit chemotherapy-induced gonadotoxicity. In addition, multiple injections could damage the testis, as shown in a rodent model where repeated injections with fine needles resulted in severe damage and tubular atrophy (Shufaro *et al.*, 2002). Nanotechnology for tissue targeted drug delivery and controlled release could be used to deliver cytoprotective agents to the prepubertal testis. This could be useful when considering agents that have a short-half life or low solubility. Nano-based therapies involve utilization of nanostructures such as nanoparticles or nanorobots to deliver drugs. Such nanostructures are based on metallic, organic, inorganic or polymer compounds, for example liposomes, micelles and dendrimers. These nanoparticles can easily diffuse into the body, with reported increased bioavailability. Targeted delivery can be achieved through addition to the surface of nanostructures specific antigens, proteins or lipids that are expressed on cellular membranes within

the targeted cells/tissue (reviewed in Patra *et al.*, 2018). For example, Sertoli cells could be targeted by the FSH hormone or through other BTB specific proteins. Alternatively, magnets could be used to draw metallic nanostructures to the testis (reviewed in Snow-Lisy *et al.*, 2011). Nanotechnology has also been used to develop anticancer drug-delivery systems to improve targeting of chemotherapy agents to tumours, rather than healthy tissues. Liposomal formulations of chemotherapy drugs vincristine or doxorubicin are FDA-approved for inclusion in cancer treatment (Quarello *et al.*, 2012; Soosay Raj *et al.*, 2013). The biology behind these liposomal formulations of chemotherapy drugs are that lipophilic or hydrophilic agents are contained within liposomes and therefore do not undergo degradation in the blood stream. These liposomes accumulate within tumours due to the enhanced permeability and retention effect. This occurs as a result of changes in the vasculature of tumours, which are more 'leaky' due to gaps forming between epithelial cells. In addition, cells within the tumours are generally less densely packed, with larger extracellular volume. This results in accumulation of the liposomal drugs within the tumour rather than in healthy tissues, with these drug formulations having different pharmacokinetics compared to the drug alone (reviewed in Massing and Fuxius, 2000; Yingchoncharoen *et al.*, 2016).

7.3 Models to Study Chemotherapy-induced Testicular Toxicity

Within this thesis, two *in vitro* animal model systems were used to assess chemotherapy-induced gonadotoxicity; a cell line model and a tissue culture model both derived from rodent tissues. From the data collected during this PhD, comparisons can therefore be made in regards to use of cell line or tissue culture models. In addition, further comparisons can be made when comparing *in vitro* to *in vivo* models, as well as use of rodent animal models compared to humans.

7.3.1 In Vitro vs In Vivo

Culture models were used during this PhD to assess gonadotoxicity of chemotherapy agents; this required a number of considerations upon designing these experiments and interpreting the results. When using culture systems for toxicity testing, drug metabolism should be considered. Many chemotherapy drugs are metabolized within the liver to activate the drug, for example, cyclophosphamide is metabolized forming active metabolites including phosphoramide mustard and acrolein. These active metabolites are therefore used in culture to determine the toxicity of such chemotherapy agents (for examples see Lopes *et al.*, 2016; Smart *et al.*, 2018). The chemotherapy drugs investigated within this study, however, do not require liver metabolism to form active metabolites, therefore further considerations were not required when designing experiments. Of the drugs examined, there is little data in regards to cisplatin metabolism, with aquation taking place directly within the cell to form the 'active metabolite' (see 3.2). Metabolism of doxorubicin occurs directly within cells through deglycosylation and carbonyl-reduction reactions, as shown in cardiomyocytes (Licata *et al.*, 2000). In comparison, liver metabolism by cytochrome P450 isoenzymes inactivates vincristine (Zhou-Pan *et al.*, 1993). Ultimately, all drugs are deactivated and eliminated from the body. Within our *in vitro* model systems, the cells and tissues are exposed to the chemotherapy agents for 48 and 24hrs, respectively. The activity of these agents *in vitro*, however, is unknown and this makes it difficult to compare *in vivo* to *in vitro* models.

Another issue arising from use of culture systems is the relevance of drug concentrations used *in vitro* to clinically relevant concentrations *in vivo*. The maximum doses (C_{max}) of paediatric serum levels of chemotherapy agents and, due to the lack of studies in children, adult serum levels of antioxidants following supplementation were used as a guide when selecting *in vitro* concentrations to determine dose-responses in our culture models. Such serum level concentrations, however, may not reflect the concentrations to which the testes are exposed to *in vivo*. Indeed, the testis could be exposed to higher or lower doses than stated serum

levels depending on multiple factors, including the half-life of the drug, bioactivity, drug elimination as well as bioaccumulation within the testis, which all can differ from person to person. Determining intratesticular concentrations of drugs is not feasible in human patients, particularly children, due to ethical concerns as well as the potential damage that could be caused to the testis from sampling. *In vitro* concentrations may not reflect *in vivo* concentrations and this needs to be considered when performing such cultures as those undertaken within this PhD. This is of importance when considering the results of antioxidant studies, since protective effects were observed at higher antioxidants concentrations than those typically found in serum (Chapter 6). In addition, dose-dependent effects of vincristine in the prepubertal mouse testis were noted from 0.1µg/ml and above, which may be higher than serum levels as reported in children (see 4.4.3, Crom *et al.*, 1994; Liston and Davis, 2017) (Chapter 4). This could account for the difference between *in vitro* studies and patients. In addition, the use of liposomal formulations to increase drug delivery can result in higher serum drugs levels, where for example maximum concentrations reported for liposomal vincristine is 1µg/ml, and for doxorubicin 8µg/ml (Liston and Davis, 2017). This is currently an unavoidable problem when using culture techniques; however, it is an important consideration when analysing data such as the data gathered during this PhD.

In vivo experiments with animal models could be performed as an alternative to culture methods, where for example the animals would be treated with doses of agents that are given to children. These doses are normally calculated in relation to the surface area or weight of the patient and therefore could be extrapolated for use with rodents. The gonadotoxicity can then be assessed within the rodent testis or with xenograft models with use of human testis samples. This approach may be considered more physiologically relevant since drug parameters such as half-life, bioactivity, tissue retention, clearance etc. would be preserved, albeit in a rodent model; however, the use of culture systems enable reductions in the number of animals used for research, in keeping with the 3Rs (reduce, replace and refine)

(Brannen *et al.*, 2016). In addition, young neonatal mice treated with chemotherapy agents do not fare well and might be at greater risk of dying prematurely. A step-wise approach to toxicology studies is currently considered best practise, where for example initial studies are undertaken with *in vitro* models followed by validation with *in vivo* models.

7.3.2 Cell Line vs Tissue Culture

There are advantages and disadvantages of using either cell line or tissue culture models. Within this thesis, the decision to carry out research with the GC-6spg cell line model was initially made in order to examine ROS production with use of a fluorogenic probe, which had not been used/optimized in a tissue culture model before. The plan was therefore to use them in experiments with a cell line and to then optimise their use in the tissues. Due to time constraints however, further experimentation with this probe in tissue could not be undertaken. Nonetheless, the value of using the cell culture models to assess gonadotoxicity and the protective capacity of antioxidants was recognized. The GC-6spg cell line is derived from adult rats with SSC characteristics and immortalized with SV40 large T antigen (van Pelt *et al.*, 2002). There are a number of immortalized testicular cell lines derived from rodents that can be used to assess the effects of chemotherapy on the testis. This includes those derived specifically from immature rodents including those representative of germ cells (GC-1spg, C18-4 and S4 cell lines), Sertoli cells (TM4, SerW3, 15P-1, SMAT1, SK11 and 93RS2 cell lines), Leydig cells (TM3 cell line) and PTM cells (TR-M) (reviewed in Rahman and Huhtaniemi, 2004; Wang et al., 2016). Primary testicular cells can also be cultured; however, germ cells do not undergo extensive proliferation *in vitro* since these cells are no longer in contact with the somatic cell niche. A major disadvantage of using cell lines is that these cells are another physiological step away from *in vivo* derived tissues, compared to cultured tissue due to the immortalization process which can occur spontaneously or be induced (Tharmalingam *et al.*, 2018). There is also tendency for isolated cells to differentiate in culture. This requires monitoring for changes in morphology, proliferation, etc. and

may require validation of the specific cell types before undertaking experiments. A major advantage of using such cell lines is the ability to quickly and relatively easily culture cells in vast numbers that can be used for testing a number of different compounds and determining the underlying molecular events in one cell type. Indeed, further work following on from this thesis could involve determining the cytotoxicity of other chemotherapy agents, such as the taxanes, since fertility risk is unknown with these agents (see table 1.1). In addition, the protective effects of other antioxidants including selenium and vitamins, identified in Yasueda *et al.* (2016) study, could be undertaken with cell culture models.

Tissue culture models are considered more physiologically relevant compared to cell lines since cells are maintained within the *in vivo* architecture to support their differentiated state and functional capacity. Indeed, tissue culture with mouse neonatal testis fragments for up to eight weeks has resulted in the formation of functional spermatozoa capable of fertilizing oocytes and producing healthy offspring (Sato *et al.*, 2011a). This study was used to design our testis tissue culture model, with tissue cultured in αMEM media supplemented with KSR. The testis culture model used here was modified from the original in the Sato *et al.* (2011a) study where instead of agar blocks, testis tissue is cultured on membranes (Lopes *et al.*, 2016). This culture model could be used to determine the molecular signals involved in spermatogenesis, since exactly how this culture media supports mouse spermatogenesis is undetermined. Research from the same group that developed the testis culture model are testing out different chemically defined media to determine which factors may be important for spermatogenesis including retinoic acid, FSH, LH and testosterone. However, to date they have been unable to promote full spermatogenesis with variations of chemically defined media (Sanjo *et al.*, 2018).

7.3.3 Animal Models vs Humans

Animal models are commonly used to model human diseases and for toxicology testing of new drugs before clinical studies, since such exploratory experiments cannot be performed on humans (reviewed in Vandamme, 2014). Species-specific differences; however, need to be considered upon interpretation of the results and include potential species differences in drug interactions, activity and elimination. Species differences in testis sensitivity and response to chemotherapy agents may also exist, since differences in the biology (for example genetics and physiology) between humans and other mammals are accepted (see 1.2 for examples in relation to testis development, and Working, 1988 for broad review). Work with animal models can only give an indication as to the potential effects/response of chemotherapy treatments/antioxidants in human samples/patients. However, the findings of similar effects of cisplatin and carboplatin following *in vitro* exposure in human fetal testis tissue at similar doses to those used in the animal study (around 0.1µg/ml) validates this animal model.

Experimental culture models utilizing human testicular cells and tissues are available, as reviewed in Tharmalingam *et al.* (2018), which have been used to study testis development and function. This includes culture of immortalized cell lines, dissociated cells, tissues or isolated seminiferous cords. Due to the limited availability of human testis tissue available for research, however, the use of animal models for assessing chemotherapy-induced testicular toxicity is an invaluable tool for such research. Currently there are no well characterized, immortalized, non-malignant human cell lines representative of non-malignant testicular cells available for research (Tharmalingam *et al.*, 2018). A human germline stem cell line and Sertoli cell line (hS1) have been derived from obstructive azoospermia patients, which have been validated for expression of cell specific markers and proliferation capacity, however differentiation of the germ cells and further characterisation are required before use (Hou *et al.*, 2015; Wen *et al.*, 2017). Tissue culture models of human adult and fetal testicular tissue have been used to study the effects of chemicals and

pharmaceutical agents such as phthalates and analgesics (reviewed in Tharmalingam *et al.*, 2018; Kilcoyne and Mitchell, 2019b). However, to date, research using prepubertal human testis tissue has focused on development and function of the testis, since there is still little research specifically into human testis development. Studies examining the impact of chemotherapy on the fetal and prepubertal testis are underway, including research by Professor Rod Mitchell's group (Melissa Tharmalingam & Gabrielle Matilionyte, revised manuscript currently under consideration by BMC Medicine). Research has been hampered by the lack of culture methodologies that support full spermatogenesis *in vitro*. Current research has been able to produce sperm-like cells *in vitro* from cultured prepubertal tissues but have yet to show functional competency of these cells (De Michele *et al.*, 2016, 2018; Abofoul-Azab *et al.*, 2018). However, recent research has reported complete spermatogenesis, with formation of spermatids from 50-day cultures of 12-19 week human fetal testicular tissue (Yuan *et al.*, 2020). Germ cells were shown to undergo mitosis, meiosis and spermiogenesis where *in vitro* derived spermatids were capable of fertilization and producing embryos up to 8-cell stage, with a similar success rate as *in vivo* derived spermatids. These studies open up the possibility of using human tissue for toxicology research in the future.

7.3.4 Innovative culture methods

Alternative tissue culture/3D models of testis development using human and rodent tissues/cells are available, and include testis organoids, culture of testicular cells in 3D matrix and microfluidic chambers (reviewed in Alves-Lopes and Stukenborg, 2018; Richer *et al.*, 2020; Sharma *et al.*, 2020). Testis organoids have been created from isolated SSCs, Sertoli cells, Leydig cells and PMCs of deceased adults, which were cultured for up to 23 days. These testis organoids were exposed to cisplatin, doxorubicin, etoposide or busulfan for 48hrs, and dose-dependent effects were noted. The testis organoids were able to tolerate higher doses of chemotherapy agents than equivalent 2D culture models (Pendergraft *et al.*, 2017). In addition, they were able to support steroidogenesis; however, complete spermatogenesis was not

accomplished, which may be a result of the 23-day culture period since human spermatogenesis takes up to 60 days. The use of microfluidic chambers, which mimics the circulatory system thereby maintaining the homeostasis of the tissue with removal of waste etc., has been used to successfully culture neonatal mouse testis tissue for six months with production of functional sperm capable of producing offspring (Komeya *et al.*, 2016). Use of multi-organ microfluidic chips with culture of testis tissue in one chamber and liver tissue in another has been used to assess the toxicity of chemotherapy agents that require liver metabolism to become active, such as cyclophosphamide (Baert *et al.*, 2020). Alternative models for testis development may be the future of *in vitro* gonadotoxicity testing.

7.4 Future Work

Following completion of the research carried out during this PhD, there are multiple routes in regards to future work; however, in addition to those already mentioned in the relevant chapters, I believe long-term cultures with the use of the mouse prepubertal testis culture is the next logical step. *In vitro* spermatogenesis following eight weeks of culture has been previously observed in the Sato *et al.*, (2011a) paper. By performing long-term cultures, the effects on fertility can be determined following exposure to chemotherapy agents during prepubertal development. Long-term cultures had been planned here, given the results from the vincristine exposure (Chapter 4) where gonadotoxic effects were noted in short-term cultures. In addition, following observations of potential protection from antioxidant treatment with short-term cultures, it would be essential to determine whether this protection translates to fertility to determine whether the cytoprotective agent is protecting against chemotherapy-induced damage or is protecting damaged cells from undergoing cell death. Maintenance of damaged cells could affect fertility and the health of offspring, as observed following amifostine treatment to protect against doxorubicin-induced damage where later fertility was not protected (Vendramini *et al.*, 2012).

Protection of the SSC population in the testis as well as the somatic cells responsible for maintaining the stem cell niche against chemotherapy-induced damage are essential for later fertility (reviewed in Stukenborg *et al.*, 2018a). The Sato *et al.*, (2011b) study has shown the potential of culturing mouse prepubertal testis fragments and supporting transplanted SSCs within these fragments to produce functional sperm. This culture system could be of interest to determine the capability of the chemotherapy exposed SSCs or somatic cells to support spermatogenesis and the impact chemotherapy has on the competency of these cells. For example, ubiquitously expressing GFP mice and/or testis fragment from these mice could be exposed to chemotherapy agents. These chemotherapy-exposed SSCs could then be isolated from the testis and injected into untreated testes and cultured to determine whether SSCs are capable of producing spermatozoa by tracing the outcome of GFP positive SSCs. The converse could also take place, whereby healthy SSCs are injected into treated testes to determine the capability of the somatic cells to support healthy germ cells. Indeed, this may be the future of fertility preservation for cancer survivors whose fertility has been affected, whereby SSCs and/or germ cells from cryopreserved prepubertal testis fragments are auto-transplanted into the adult following chemotherapy treatment. Alternatively, research such as conducted during this PhD could identify a cytoprotective agent to reduce or even eliminate chemotherapy-induced prepubertal testicular toxicity, unless or until cancer treatments that do not induce such side effects are developed.

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Appendix A (Review publication)

REPRODUCTION
REVIEW

How does chemotherapy treatment damage the prepubertal testis?

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Abstract

Chemotherapy treatment is a mainstay of anticancer regimens, significantly contributing to the recent increase in childhood cancer survival rates. Conventional cancer therapy targets not only malignant but also healthy cells resulting in side effects including infertility. For prepubertal boys, there are currently no fertility preservation strategies in use, although several potential methods are under investigation. Most of the current knowledge in relation to prepubertal gonadotoxicity has been deduced from adult studies; however, the prepubertal testis is relatively quiescent in comparison to the adult. This review provides an overview of research to date in humans and animals describing chemotherapy-induced prepubertal gonadotoxicity, focusing on direct gonadal damage. Testicular damage is dependent upon the agent, dosage, administration schedule and age/pubertal status at time of treatment. The chemotherapy agents investigated so far target the germ cell population activating apoptotic pathways and may also impair Sertoli cell function. Due to use of combined chemotherapy agents for patients, the impact of individual drugs is hard to define, however, use of *in vivo* and *in vitro* animal models can overcome this problem. Furthering our understanding of how chemotherapy agents target the prepubertal testis will provide clarity to patients on the gonadotoxicity of different drugs and aid in the development of cytoprotective agents.

Reproduction (2018) **156** R209–R233

Introduction

The overall childhood cancer survival rate has increased substantially in recent decades, with the current 5-year survival rate at around 80%, compared to about 58% in the late 1970s (Miller *et al*. 2016). This marked advance, due in large part to improved chemotherapy treatments, has led to a growing population of long-term childhood cancer survivors. However, chemotherapy drugs do not exclusively target malignant cells, also eliciting side effects due to off-target damage to healthy tissues. Given this situation, research is increasingly focusing on preventing damage to healthy organs, to improve the quality of life for childhood cancer survivors. For younger patients, detrimental effects of treatment on fertility can be a major concern (Zebrack *et al*. 2004). This is particularly problematic for male survivors of childhood cancer, given the current lack of available fertility preservation treatments, since, unlike adult patients, these prepubertal patients do not yet produce mature spermatozoa that can be used for routine sperm cryopreservation (Wyns *et al*. 2010, Wallace 2011, Mitchell *et al*. 2017). Recently, centres have started to cryopreserve immature testicular tissue from prepubertal boys before the commencement of chemotherapy treatment. In 2015, there were seven centres in Europe collecting such tissues with more than 260 prepubertal samples stored, with biopsies undertaken only when treatment is deemed at high risk for later fertility complications (Picton *et al*. 2015, Mitchell *et al*. 2017). At this point, however, it is not yet certain if such cryopreserved tissue can be successfully used later to restore fertility in humans, as production of viable sperm from such tissue is yet to be shown. A recent report has described the generation of sperm-like cells after 3D culture of isolated spermatogonial cells obtained from testis biopsies taken from prepubertal boys undergoing chemotherapy treatment (Abofoul-Azab *et al*. 2018). This is encouraging for the future, but in this preliminary study, the technique was successful in only one out of six patient biopsies, and the functionality of the sperm-like cells remains to be established. Research in animal models has been more successful in showing the potential of fertility preservation techniques, which could be developed for clinical use (reviewed in Picton *et al*. 2015, Giudice *et al*. 2017). Transplantation of spermatogonial stem cells (SSCs) or frozen-thawed immature testicular tissue grafted back into the adult has been successful in producing functional gametes in animal models including the non-human primate and murine (Brinster & Zimmermann 1994, Mitchell *et al*. 2010, Hermann *et al*. 2012, Jahnukainen *et al*. 2012).

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Healthy offspring have been produced through IVF/ICSI using sperm derived from xenografted non-human primate immature testicular tissue demonstrating the potential of this technique for clinical application (Liu *et al*. 2016). There are concerns, however, for non-solid tumours that malignant cells could be reintroduced back into the patient, particularly with tissue transplantation as shown in the Hou *et al*. (2007) study where leukaemic infiltration was noted within the xenografted testicular tissue. In addition to *in vivo* techniques, sperm has been grown in culture from immature testis through *in vitro* spermatogenesis, and these sperm have been used for IVF/ICSI to produce viable embryos in a mouse model system (Sato *et al*. 2011). There have also been recent reports of *in vitro* culture of human prepubertal testicular tissue, although without completion of spermatogenesis (de Michele *et al*. 2017, 2018). For all such potential fertility preservation techniques, further research is needed to allow the methods to be successfully transformed for use with human tissue, after which further validation will be required to ensure that such methodologies are efficient and safe for clinical use. An alternative strategy, rather than the subsequent restoration of fertility after chemotherapy-induced infertility, would be the development of interventions administered before and/or during chemotherapy treatment to prevent the damage from occurring in the first instance, thus protecting the fertility potential of the patient. Cytoprotective agents that specifically protect the prepubertal testis against chemotherapy-induced damage, without interfering with the toxicity to cancer cells, could potentially be employed as part of the chemotherapy treatment regimen. However, further research is required to fully understand how chemotherapy drugs target the prepubertal testis and which compounds could potentially prevent such damage.

This review provides an overview of research to date that has focused upon the direct, chemotherapy drug-induced damage to the prepubertal testis: such studies often rely on histological analysis. Damage can also be conferred from studies examining hormonal changes after cancer treatment. For example, AMH and inhibin B can be used as markers of Sertoli cell function, although the use of such markers during prepuberty is yet to be investigated (Dere *et al*. 2013, Stukenborg *et al*. 2018*a*). The use of hormonal indicators to determine chemotherapy-induced damage is not discussed further in this review. The review discusses papers only where they investigate effects on the prepubertal testicular tissue/cells, either from human or animal models. Determining which cell types within the prepubertal testis are directly targeted by chemotherapy drugs, along with the potential mechanism of action of the different drugs, will provide vital information enhancing our knowledge on the gonadotoxicity of chemotherapy agents and the development of cytoprotective agents against chemotherapy-induced damage.

Methodology

A literature search was conducted to identify relevant papers to query our research question; how does chemotherapy treatment damage the prepubertal testis? A review of the literature was performed using PRISMA guidelines (Moher *et al*. 2009). Relevant papers were identified using PubMed to search for appropriate references, searching key words including prepubertal/immature, testes, chemotherapy (including classes of drugs) and fertility preservation. Additional references were found by searching reference lists of such papers. The abstracts of identified papers were screened for relevance in relation to chemotherapy treatment during prepuberty and impact on the testis. The eligibility of relevant studies was assessed by reading the screened papers in full to ensure that all those included in this review are all concerning prepubertal tissues, whether from human patients or animal models. Clinical studies are included where the histology of the testis of human patients following prepubertal cancer treatment is examined during or at the end of the treatment period or during adulthood. *In vivo* studies in animal models where drug exposure occurred prior to the onset of puberty as well as *in vitro* studies of cultured cells and tissues obtained from prepubertal animals were also analysed. Potential fertility cytoprotectants were included where research was performed on prepubertal/immature subjects. Of the papers that were excluded, the majority were due to chemotherapy treatment taking place during/after puberty, analysis of chemotherapy-induced damage through hormonal changes or failure to report the dose of the chemotherapy agents. Overview of our research strategy is shown in Fig. 1.

Childhood cancer and treatment

Incidence of childhood cancer

Each year around 1800 children are diagnosed with neoplastic disease, accounting for 1% of new cancer diagnoses in the United Kingdom (Wallace 2011, Cancer Research UK 2015). Cancer is the second most common cause of death in children. The nature of cancers that develop at this young age differs from those of adult malignant tumours. In infants, tumours of embryonal origin such as neuroblastomas are the most frequent cause, whereas in older children, leukaemia (particularly acute lymphoblastic leukaemia) as well as central nervous system tumours and lymphomas (non-Hodgkins and Hodgkins) are more often diagnosed. There is also a sex difference in incidence, with boys 1.2 times more likely to be diagnosed with cancer,

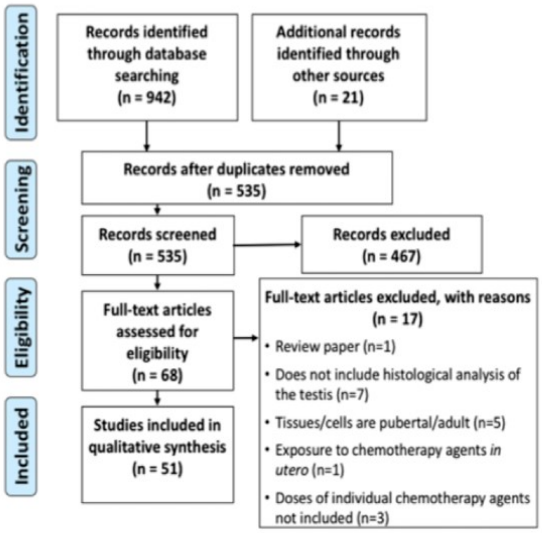


Figure 1 PRISMA flow diagram of literature search. PRISMA flow diagram of search results, study screening, and study inclusion, following a review of the literature carried out using PRISMA guidelines (Moher *et al.* 2009).

particularly lymphomas and central nervous system tumours (Kaatsch 2010, Kelly 2017).

Chemotherapy treatment

Chemotherapy and radiotherapy are a mainstay of treatment for childhood malignancies. For chemotherapy drugs, the refinement and use of combined drug treatments have contributed greatly to the dramatic increase in childhood cancer survival rates over recent decades (Anderson *et al.* 2015, Miller *et al.* 2016). Multiple different drugs have been established in treatment regimens; these kill cancer cells through various mechanisms, often targeting proliferating cells (Table 1) (Malhotra & Perry 2003, Lind 2011). The gonadotoxic impact and relative risk of later infertility of individual chemotherapy agents included in treatment regimens against childhood malignancies can at present only be estimated, since the risk is dependent on multiple factors including dosage, treatment length, age at time of treatment and the sensitivity of individual patients to chemotherapy treatment. The current classification of chemotherapy drugs and the dosages considered to result in infertility are outlined in Table 1; however, these classifications/dosages are debated. Given that clinicians use this knowledge to determine whether or not to offer cryopreservation of testicular tissue before commencement of treatment, further elucidation on relative risk/gonadotoxicity is urgently required. At present, alkylating and alkylating-like agents such as cyclophosphamide and cisplatin are considered to be highly gonadotoxic with these agents intercalating into DNA, disrupting basic cellular processes. These agents are commonly included in treatment regimens to treat a wide range of childhood malignancies and are known to result in subsequent infertility in adulthood (Chow *et al.* 2016). Agents from alternative drug classes which have different mechanisms of action are considered low or moderate risk of later infertility and include; antimetabolites such as cytarabine, vinca alkaloids e.g. vincristine and topoisomerase inhibitors including etoposide. There are also some chemotherapy agents, including taxanes, where infertility risk/gonadotoxicity is at present unknown. Further details are included in Table 1.

For the vast majority of paediatric cancers, combined chemotherapy with multiple agents is required to effectively treat the disease, with commonly used combinations including MOPP (nitrogen mustard, vincristine, procarbazine and prednisolone) or ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) for treatment of Hodgkins lymphoma and CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) for non-Hodgkins lymphoma (Corrie 2011). The administration of several agents in a treatment regimen could potentially result in additive or even multiplicative side effects on healthy tissues.

Testis development

The testis is responsible for producing mature spermatozoa along with the main male reproductive hormone, testosterone. During prepuberty, the testis was originally thought to be relatively inactive based on studies demonstrating few morphological changes and a lack of hormone production during this period (Rey 1999). However, further detailed analysis has shown that the prepubertal testis undergoes important developmental processes, which are required for normal adult functioning (Fig. 2A). This section will outline what is currently known regarding testis development, focusing primarily upon human development. Many studies, however, have relied upon animal models to observe prepubertal changes due to the challenges of studying the human testis; information about non-human species will be specified where relevant.

Foetal life

The testis forms during early foetal life from an undifferentiated bipotential gonad. The primordial germ cells, originally located outside the embryo within the yolk sac, migrate and populate the gonadal ridge (Stukenborg *et al.* 2014). Under the action of the *SRY* (sex-determining region Y) gene, which drives production of the SOX9 (SRY-box 9) protein, Sertoli cells differentiate from precursors cells within the gonadal ridge and engulf the primordial germ cells which are now classified as gonocytes. This configuration results in the formation of seminiferous cords, which predominantly

Table 1 Overview of commonly used chemotherapeutic agents in childhood cancer treatment regimens.

Chemotherapy drug class	Childhood cancer usage	Mechanism of action	Cell cycle phase	Compounds	Current prediction of infertility risk*
Alkylating and alkylating-like agents	Bone cancer CNS tumours Hodgkins lymphoma Kidney cancer Leukaemia Neuroblastoma Non-Hodgkins lymphoma Soft tissue sarcoma	Alkyl groups intercalate into nucleic acids and proteins. Intercalate into DNA by binding to the guanine or cytosine bases, resulting in crosslinks which disrupt DNA replication/transcription	Non-specific	Carboplatin Chlorambucil Cisplatin Cyclophosphamide Ifosfamide Mechlorethamine Melphalan Oxaliplatin Procarbazine	Moderate High (>1.4 g/m^2) High (>0.6 g/m^2) High (>7.5 g/m^2) High (>60 g/m^2) High High (0.14 g/m^2) Moderate High (>4 g/m^2)
Anthracyclines	Bone cancer Hodgkins lymphoma Kidney cancer Leukaemia Neuroblastoma Non-Hodgkins lymphoma Soft tissue sarcoma	Target topoisomerase II, intercalate into DNA and produce free radicals	M-phase	Daunorubicin Doxorubicin	Unknown Moderate
Antimetabolites	Bone cancer Leukaemia Non-Hodgkins lymphoma	Disrupt DNA/RNA synthesis	S-phase	Cytarabine Fluorouracil Mercaptopurine Methotrexate Thioguanine	Moderate Unknown Low Low Unknown
Non-Anthracycline Antibiotics	Bone cancer Hodgkins lymphoma Soft tissue sarcoma	Multiple mechanisms of action including DNA intercalation, disruption of DNA synthesis and DNA fragmentation	Non-specific	Bleomycin Dactinomycin Mitomycin	Low Low Unknown
Taxanes	Ewing sarcoma	Inhibit disassembly of microtubules	G_2/M-interphase	Docetaxel Paclitaxel	Unknown Unknown
Topoisomerase inhibitors	Bone cancer Leukaemia Soft tissue sarcoma	Target either topoisomerase I or II to disrupt DNA replication	M or S-phase	Etoposide Irinotecan Teniposide Topotecan	Low Unknown Unknown Unknown
Vinca alkaloids	Bone cancer CNS tumour Hodgkins lymphoma Kidney cancer Leukaemia Neuroblastoma Non-Hodgkins lymphoma Soft tissue sarcoma	Inhibit assembly of microtubules	M-phase	Vinblastine Vincristine	Low Low

*Currently considered risk of infertility adapted from Wyns *et al.* (2010).

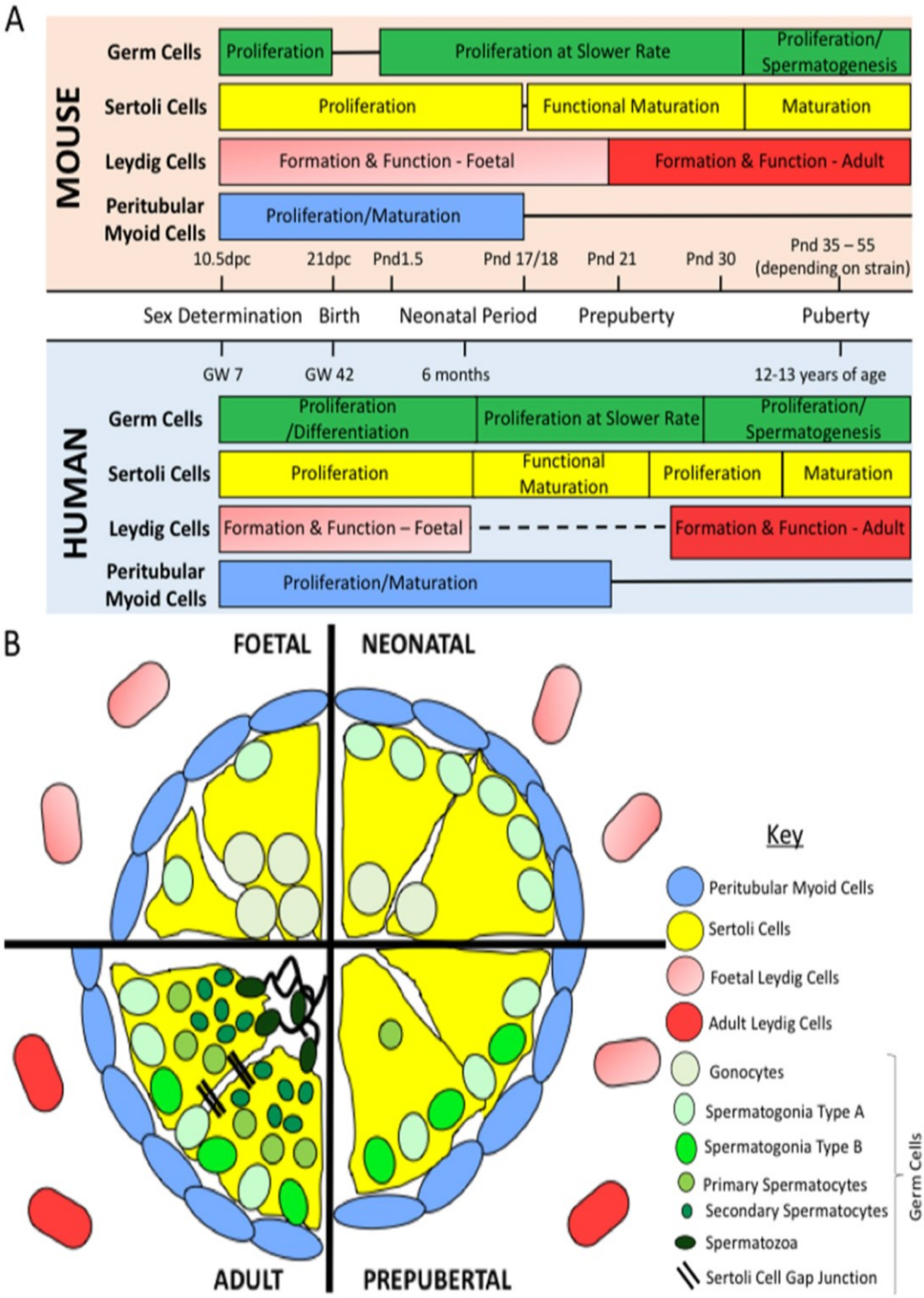


Figure 2 Comparison of testicular development in humans and rodents. (A) Relative timeframe of important developmental processes taking place between foetal development and puberty in humans (Chemes 2001) and the mouse model (Vergouwen *et al.* 1993). Solid line indicates no activity of the cells at the relevant time points and dashed line represents the unknown nature of Leydig cell development during this timeframe. (B) Comparison of the histology of the testis throughout development in the human, from foetal development through to the adult testis. dpc, days post coitum; GW, gestational week; pnd, postnatal day.

contain Sertoli cells with centrally located gonocytes. The Sertoli cells are key drivers in the differentiation and function of other cellular components of the testis including the Leydig cells and peritubular myoid cells (reviewed in Svingen & Koopman 2013) (Fig. 2B). The hypothalamic–pituitary–gonadal axis (HPG) is active during foetal life, producing the gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone, which are essential for the formation, maturation and function of key somatic cells including the Sertoli and Leydig cells (Stukenborg *et al.* 2018*a*).

During foetal development, the gonocytes actively proliferate into early neonatal life, and differentiation of the gonocytes into pre-spermatogonia occurs asynchronously during foetal and early postnatal life, with the gonocytes having greater proliferative activity than the pre-spermatogonia. In the rodent model, however, the proliferation of the gonocytes ceases during late foetal life and resumes during the early postnatal period (Ferrara *et al.* 2006, Mitchell *et al.* 2008, Wu *et al.* 2009). Sertoli cells actively proliferate during foetal development under the control of multiple hormones, particularly FSH. This period of proliferation is very important in determining adult numbers of Sertoli cells (Sharpe *et al.* 2003). Peritubular myoid cells develop from interstitial cells, when multiple layers of these cells along with extracellular matrix proteins surround and form the wall of the seminiferous cords. These cells contribute to the basement membrane providing structural support during testis development and will function in the adult to aid movement of mature sperm towards the lumen (Mayerhofer 2013, Svingen & Koopman 2013). The endocrine somatic Leydig cells are situated within the interstitial compartment between seminiferous cords and are composed of different cell populations throughout an individual's lifespan. The foetal Leydig cells (FLCs) produce testosterone throughout foetal life, which is essential for masculinization of the male reproductive system. These cells ultimately regress after birth and are replaced by adult Leydig cells (ALCs) during puberty (Habert *et al.* 2001, Chen *et al.* 2009, Svechnikov *et al.* 2010), however, the presence and/or function of the FLCs have recently been shown to be important in the formation and function of the ALCs in a rodent model (Su *et al.* 2018). Whether FLCs and ALCs are distinct populations of cells or share a common stem/progenitor cell lineage is at present unknown; however, stem ALCs have been observed in the human foetal testis (Kilcoyne *et al.* 2014, Teerds & Huhtaniemi 2015).

Neonatal life

During the first 3 months of neonatal life, the reproductive system undergoes a short period of activity known as 'mini-puberty', where the HPG axis is active (Kuiri-Hänninen *et al.* 2014). The origin of the Leydig cells that produce testosterone during 'mini-puberty' is unknown, possibly forming from precursor cells or from the FLCs (Svechnikov *et al.* 2010). The function of 'mini-puberty' is yet to be clarified, but is thought to be linked to later reproductive function in the adult (Copeland & Chernausek 2016). This short neonatal activity has also been observed in the primate animal model during neonatal development but does not appear to occur in rodents, where infancy overlaps with early stages of pubertal development (Chemes 2001, Kelnar 2002).

The volume of the testis increases six-fold during neonatal development due to proliferation of Sertoli cells stimulated by FSH secretion; 93–95% of the seminiferous cord mass is attributed to the Sertoli cells at this stage (Müller & Skakkebaek 1983, Rey *et al.* 1993; reviewed in Petersen & Söder 2006). This increase in cell number results in an overall increase in seminiferous cord length but not diameter, as each Sertoli cell maintains contact with the basement membrane, the cells spreading lengthwise (Chemes 2001). Therefore, in a cross-sectional area of the seminiferous cord, it may appear as though the cell density remains stable or even decreases during this period of development, which can be misleading. The resulting number of Sertoli cells will ultimately determine sperm production in the adult, with each Sertoli cell able to support a species-specific number of germ cells (Sharpe 2001).

During the foetal and early postnatal period of development, centrally located gonocytes within the seminiferous cords differentiate and lose their pluripotency. At around 2–3 months of age, the differentiating cells migrate towards the basement membrane, developing into the SSCs (Culty 2009). The SSCs are now located within the stem cell niche in the basal compartment of the seminiferous cords, outside of the blood–testis barrier (BTB), which will later form during puberty to protect post-meiotic germ cells (Stanton 2016, Li *et al.* 2017). The maintenance of the SSC niche is dependent on factors produced by somatic cells (Stukenborg *et al.* 2018*a*). In rodents, there is a relatively rapid and synchronous differentiation of gonocytes to pre-spermatogonia and migration to the basal membrane occurring by postnatal day (pnd) six (Mitchell *et al.* 2008, Wu *et al.* 2009).

Prepuberty

Prepuberty lasts for around 11 years until the HPG axis is once again reactivated and the child enters puberty. During prepuberty, perceived reduced cellular activity in the testis was thought to confer protection against insult (Rivkees & Crawford 1988) (Fig. 2A). However, this does not appear to be the case as shown in studies demonstrating infertility in adults following childhood chemotherapy treatment (Chow *et al.* 2016).

During prepuberty, the proliferative rate of the germ cell population is reduced in comparison with foetal development; nevertheless, there is a three-fold

increase in the overall germ cell population during this time period (Müller & Skakkebaek 1983). At this point, the SSC pool is represented by undifferentiated A spermatogonia composed of two populations of cells with a dark (A_{dark}) or pale (A_{pale}) appearance. A_{dark} spermatogonia are believed to represent the reserve stem cells with low mitotic activity, whilst the A_{pale} cells are actively proliferating. Upon differentiation, the A_{pale} spermatogonia differentiate to B spermatogonia, which can be observed from 4 to 5 years of age (Paniagua & Nistal 1984). Type B spermatogonia will enter meiosis to form spermatozoa in the adult (Ehmcke *et al.* 2006, Stukenborg *et al.* 2014). In rodents, A_{single} (A_s) spermatogonia actively proliferate to form two conjoined daughter cells known as A_{paired} (A_{pr}) cells which continuously divide forming $A_{aligned}$ (A_{al}) chains (reviewed in de Rooij & Russell 2000, Ehmcke *et al.* 2006). Occasionally, spermatogonia enter meiosis, leading to the infrequent observation of primary spermatocytes during prepuberty; however, these cells quickly degenerate since the somatic cells are not mature enough to support full spermatogenesis at this stage (Chemes 2001). The diameter of the tubules is unaltered during prepubertal development, with the lumen expanding only later during puberty due to intense germ cell proliferation that results in the expansion in tubule width (Chemes 2001). Reference values based upon a systematic review and meta-analysis performed by Masliukaite *et al.* (2016) indicates that spermatogonia number per tubular cross-section and density per area (cm^3) decrease during the first 3 years of life followed by a gradual increase up to 6 to 7 years of age, plateauing up to age 11 when boys begin to enter puberty and numbers increase dramatically. The initial decrease may be a result of programmed cell death of the gonocytes that failed to migrate to the basement membrane earlier in development (Masliukaite *et al.* 2016).

The Sertoli cells in the prepubertal testis appear morphologically immature, with little cytoplasm and with the nuclei arranged in palisade formation with small nucleoli (Chemes 2001). These somatic cells differentiate and undergo functional maturation during prepuberty, with increased expression of androgen receptors and connexin 43, as well as expression of vimentin and inhibin β markers (Brehm *et al.* 2006, de Michele *et al.* 2018). In addition, Sertoli cells display aromatase activity and produce oestrogen during prepuberty (Chemes 2001). AMH, in particular, is secreted in large amounts in prepubertal boys and can be used as an indicator of Sertoli cell number and function; however, levels will decline during puberty and will be low throughout adulthood (Rey 1999). As in the foetus, Sertoli cell factors are involved in controlling the development/proliferation of other testicular cell types. The peritubular myoid cells proliferate and develop during early postnatal development in the rodent, under the control of FSH, with Sertoli cells having an important role in maintaining their differentiated state (Chemes 2001, Nurmio *et al.* 2012, Rebourcet *et al.* 2014). However, this dependency is lost when the peritubular myoid cells terminally differentiate during prepuberty, as shown in the rodent model (Rebourcet *et al.* 2014). These cells gain the ability to contract during puberty, with testosterone stimulating the expression of smooth muscle actin in the primate model (Mayerhofer 2013). The development of the Leydig cell population during the prepubertal period is less well understood with much of our knowledge based on rodent studies (Chen *et al.* 2009). In the rodent, Sertoli cells have an important role in stimulating ALC differentiation and will ultimately determine the number of ALCs in the adult (Rebourcet *et al.* 2014). The ALC population forms during puberty following reactivation of the HPG axis from stem/progenitor cells, which proliferate during early postnatal life (Chen *et al.* 2009).

Evidence of chemotherapy-induced direct damage to the prepubertal testis

Understanding the specific mechanisms by which different classes of chemotherapy drugs directly target and damage the prepubertal testis is essential to aid development of protective strategies. Damage induced by chemotherapy treatment can have a major impact on the patient's reproductive outcome in later life, with impaired development of sexual characteristics and potential fertility consequences (Frederick *et al.* 2016). Long-term fertility depends on continued survival of male germ cells, specifically SSCs, and of functional supporting somatic cells (Zohni *et al.* 2012, Yoon *et al.* 2017, Stukenborg *et al.* 2018*a*). However, research focusing on direct damage to the testis is lacking within a clinical setting since testis tissue biopsy is not routinely performed before or after chemotherapy treatment. With recent focus on cryopreserving prepubertal testis samples before the onset of cytotoxic treatment for potential fertility preservation in the future, more tissue is becoming available for research and therefore studies using such tissue should be more common in the future. Indeed, a recent report by Stukenborg *et al.* (2018*b*) has histologically examined testis biopsies from prepubertal patients who were selected for cryopreservation of tissue due to the cytotoxic nature of their cancer treatment regimens. Research from animal studies has the potential to aid in understanding gonadal toxicity of individual drugs and their mechanism of action, as well as to examine the impact of clinically relevant combined treatments; however, to date, there have been few such studies. The human and animal studies discussed in this review focus on chemotherapy treatment delivered during the prepubertal period, where the damage can be assessed after treatment or implied from subsequent analysis of the adult testis.

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Table 2 Human studies reporting cyclophosphamide-induced gonadotoxicity: assessment of immediate testicular damage.

			Male patients		Effect of cyclophosphamide				
Cyclophosphamide dosage	**Other drugs co-administered**	**Treatment length**	*n*	Age (years)	Testicular histology	Number of germ or somatic cells/TFI	Interstitial fibrosis/basement membrane thickening	Testicular size	**Reference**
Grams/kg body weight									
0.002 g/kg/day	Unknown	3 months	1 (prepubertal: 1; CYC treated: 1)	6	Abnormal, atrophic tubules	SCOs	Present	Small	Hyman and Gilbert (1972)
0.003–0.024 g/kg/day	Unknown	<50–400 days	7 (prepubertal: 7; CYC treated: 7)	3–11	Normal	N/A	N/A	N/A	Arneil (1972)
0.475–0.846 g/kg	Asparaginase Cytarabine Doxorubicin Mercaptopurine Methotrexate Prednisolone Vincristine	2–6 years	46	0.08–13	Abnormal	Reduced TFI >0.130 g/kg CYC. Normal TFI intermittently treated higher cumulative CYC. Poorly developed somatic cells younger patients	N/A	N/A	Ise *et al.* (1986)
Grams/area									
0.12–8.5 g/m^2	Asparaginase Bleomycin Carmustine Cytarabine Dacarbazine Dactinomycin Daunorubicin Doxorubicin Fluorouracil Lomustine Mercaptopurine Methotrexate Mitomycin C Nitrogen mustard Prednisone Procarbazine Teniposide Vinblastine Vincristine Vindesine	0.08–1 year	32 (prepubertal: 21; CYC treated: 13)	<11	Abnormal when treated with multiagent regimen including CYC	N/A	N/A	N/A	Matus-Ridley *et al.* (1985)
0.5–1.2 g/m^2	Asparaginase Cytarabine Daunorubicin Doxorubicin Mercaptopurine Methotrexate Prednisone Vincristine	1.2–88.8 months	10 (prepubertal: 8; CYC treated: 2)	9.4–16.6	N/A	Complete loss of GCs CYC treated	N/A	N/A	Müller *et al.* (1985)

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Cyclophosphamide dosage	Other drugs co-administered	Treatment length	Male patients			Effect of cyclophosphamide			Reference
			n	Age (years)	Testicular histology	Number of germ or somatic cells/TFI	Interstitial fibrosis/basement membrane thickening	Testicular size	
>1 g/m^2	Asparaginase Cytarabine Doxorubicin Mercaptopurine Methotrexate Prednisolone Vincristine	12–77 months	44 (prepubertal: 27)	3.5–15	N/A	Reduced TFI (<40%) >1 g/m^2 CYC	Present	N/A	Lendon *et al.* (1979)
3–16 g/m^2	Asparaginase Cytarabine Dexamethasone Doxorubicin Mercaptopurine Methotrexate Prednisolone Thioguanine Vincristine	~1.8 years	37 (prepubertal: 37; CYC treated: 16)	1.1–16.1	N/A	Depletion of spermatogonial pool with reduced TFI (19%)	N/A	N/A	Poganitsch-Korhonen *et al.* (2017)
<4.8 g/m^2	Asparaginase Cytarabine Daunorubicin Hydroxyurea Lomustine Methotrexate Prednisolone Thioguanine Vincristine	3–4 years	25 (prepubertal: 24)	1.23–12.35	Abnormal	Complete loss or depletion of GC pool. Normal SC	N/A	Small	Quigley *et al.* (1989)
6.2–11.4 g/m^2	Asparaginase Cytarabine Doxorubicin Mercaptopurine Methotrexate Prednisolone Teniposide Vincristine	6.6–7.6 years	23 (prepubertal: 23; CYC treated: 6)	2.8–8.6	N/A	Depletion in SSCs (↓ CD9 and OCT4) and more differentiated spermatogonia (↓ MAGE4). Recovery noted	N/A	N/A	Nurmio *et al.* (2009*a*)
8–16.8 g/m^2	Asparaginase Cytarabine Daunorubicin Dexamethasone Enocitabine Hydrocortisone Mercaptopurine Methotrexate Prednisolone Vincristine	Unknown	12 (CYC treated: 7)	1–12	N/A	Reduced TFI (<50%) with morphological changes to GCs and SCs but not linked to CYC	Present	N/A	Kobayashi *et al.* (1996)

(*Continued*)

Table 2 Continued.

Cyclophosphamide dosage	Other drugs co-administered	Treatment length	Male patients		Effect of cyclophosphamide					Reference
			n	Age (years)	Testicular histology	Number of germ or somatic cells/TFI	Interstitial fibrosis/basement membrane thickening	Testicular size		
Total dose										
1.4–20.8 g	Asparaginase Cytarabine Doxorubicin Mercaptopurine Methotrexate Prednisolone Vincristine	~4.4 years	37 (CYC treated: 14)	1.6–14.3	Abnormal	GC damage and reduced TFI (<50%)	N/A	N/A		Wallace *et al.* (1991)
7.79 g	Prednisolone	180 days	1 (prepubertal: 1; CYC treated: 1)	3–4	Normal	N/A	N/A	Normal		Berry *et al.* (1972)

Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient's sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.
[a]Information not included in study.
CYC, cyclophosphamide; GC, germ cell; SC, Sertoli cell; SCO, Sertoli cell-only tubule; SSC, spermatogonial stem cell; TFI, tubular fertility index.

Human studies

Studies examining the direct testicular damage induced following chemotherapy treatment of prepubertal human patients are few and far between, with most of these linking the damage to cyclophosphamide treatment, although in almost all cases there is co-administration of other chemotherapy drugs (Table 2). Methods of analysis are descriptive in nature following histological analysis of testicular biopsies, for example, describing the general appearance of tubules and density of germ and somatic cells present. Additional analyses include calculation of the tubular fertility index, which represents the percentage of seminiferous tubules that contain spermatogonia (Ise *et al.* 1986). To the best of our knowledge, there are no papers where chemotherapy agents were tested for cytotoxicity through *in vitro* culture of testicular cells or tissues from human patients to include in this review. Many of the included studies present limitations such as small patient cohorts, lack of universal methods for defining pubertal status and lack of adequate control groups for comparison.

Assessment of immediate testicular damage in prepubertal patients

Following on from initial histological observations published in early case reports (Arneil 1972, Berry *et al.* 1972, Hyman & Gilbert 1972), larger studies indicate a relationship between use of alkylating agents in treatment regimens and testicular tissue damage (Poganitsch-Korhonen *et al.* 2017, Stukenborg *et al.* 2018*b*). In particular, the inclusion of the drug cyclophosphamide for cancer treatment during the prepubertal period has been linked to resulting testicular damage (Table 2). The studies included here under 'immediate assessment' varied in the timeframe of analysis with the testicular damage examined at different time points including during, just before the cessation or at the end of the treatment period as well as up to a year after the end of treatment. These investigations have indicated that cyclophosphamide treatment is associated with testicular damage in a dose- and time-dependent manner. Treatment can reduce the overall size of the testis where there is depletion of the germ cell population, resulting in Sertoli cell-only tubules, as well as interstitial fibrosis and basement membrane thickening (Hyman & Gilbert 1972, Hensle *et al.* 1984, Uderzo *et al.* 1984). A cut-off dose at which such damage is evident is hard to define, since comparison between the few available studies is challenging due to the limitations previously described, as well as the variability of treatment regimens. The length of the treatment regimens may also determine the severity of the impairment, with higher cumulative doses over a shorter period of time reducing chemotherapy-induced damage (Ise *et al.* 1986). In many of the studies listed in

Table 2 and discussed further in subsequent sections, only a subset of the patients exhibit severe damage to the testis following cyclophosphamide treatment. This indicates a degree of variability/susceptibility to damage which could be due to several factors, including age and genetic predisposition.

The testis itself is composed of somatic and germ cells, which could potentially each have different sensitivities to chemotherapeutic drugs. Damage to the somatic cells during chemotherapy treatment could negatively impact on the germ cells and vice versa (Stukenborg *et al.* 2018*a*). Nurmio *et al.* (2009*a*) have reported that cyclophosphamide targets both SSCs and more differentiated spermatogonia in the prepubertal testes, as indicated by changes in gene expression of specific spermatogonial markers (MAGE A4 and CD9). This is in agreement with most papers that reported effects on germ cells, with one study describing the appearance of immature Leydig and Sertoli cells following cyclophosphamide treatment (Ise *et al.* 1986).

Assessment of the effect of prepubertal drug exposure damage in the adult

Insult to the prepubertal testes following chemotherapy treatment can be inferred from examination of pubertal/adult patients who were treated as children and can also determine the potential for the testes to recover and later undergo active spermatogenesis. Short-term analysis (1 to 5 years) and long-term analysis (6 to 10 years) following the cessation of cyclophosphamide treatment have shown that damage is often still observable in a dose- and time-dependent manner (Table 3). Patients receiving relatively high doses of cyclophosphamide have exhibited severe testicular damage with Sertoli cell-only tubules present up to 9 years after treatment (Penso *et al.* 1974, Aubier *et al.* 1989). The length of treatment regimen may also influence the disruption caused to the prepubertal testes, as shown in Etteldorf *et al.* (1976) (Table 3). However, such differences may ultimately be the result of higher cumulative doses or the age of the patient when treated, with younger patients potentially more at risk of reduced tubular fertility index and poor development of Sertoli and Leydig cells, as has been shown in Ise *et al.* (1986). Nonetheless, due to small numbers of participants, individual studies such as this can only lead to definitive conclusions when part of a larger meta-analysis. A case report has described somatic cell damage in the testis following chemotherapy during prepuberty, with the presence of immature Sertoli cells (identified by cytokeratin 18 and D2-40 markers) in a 31-year-old man treated during childhood with a regimen containing cyclophosphamide; however, causation cannot be determined from a case report (Bar-Shira Maymon *et al.* 2004).

Summary of human studies

The studies discussed above suggest that alkylating agents, in particular cyclophosphamide, can be detrimental to the prepubertal testis in a manner that can persist at least up to 10 years after the cessation of treatment. Since patients had received a combination of chemotherapy drugs, it is hard to determine the relative contributions of individual chemotherapeutic drugs to gonadal toxicity; in addition, results may have been influenced by the age/stage of pubertal development and each patient's own sensitivity to chemotherapy treatments.

Animal studies

The use of animal models has the potential to provide a clearer picture of chemotherapy drug-induced gonadal toxicity in the prepubertal context, in comparison to our very limited ability to investigate this directly in humans. Research on animal models enables researchers to administer drugs through more regulated regimens and to compare results using animals, tissues or cells. The use of animal models also opens the possibility of determining which period of development is more sensitive to chemotherapy treatment, for example by comparing infancy to prepuberty. Additionally, such studies are likely to be invaluable in determining the underlying mechanisms by which the different chemotherapy drugs damage the prepubertal testis, information which should help in the subsequent development of protective strategies designed to directly block such damage. Despite their great potential, relatively few studies have been conducted *in vivo* or *in vitro* with the majority of studies focusing on germ cell effects. Work to date has looked specifically at alkylating agents, anthracyclines, topoisomerase inhibitors, vinca alkaloids and non-anthracycline antibiotic chemotherapy treatments.

Germ cell effects

Alkylating and alkylating-like agents As in human studies, research to date using prepubertal testis of rodents points to alkylating and alkylating-like agents targeting the germ cell population in a dose- and time-dependent manner (Table 4). SSCs have been shown to be particularly sensitive to cyclophosphamide and cisplatin, with treatment inducing DNA damage *in vitro*, which could ultimately activate cell death pathways if not repaired by internal DNA repair systems (Marcon *et al.* 2010, Liu *et al.* 2014). DNA damage was noted following short-term (24 h) exposure to chemotherapeutics using an *in vitro* culture system of prepubertal (pnd 5) mouse testis (Smart *et al.* 2018). Activation of the apoptotic pathway was shown by enhanced cleaved caspase (CC) 3 expression following cyclophosphamide and cisplatin treatment with the timings differing between the agents, followed by loss of germ cells hours later. Increased numbers of apoptotic spermatogonia and

Table 3 Human studies reporting cyclophosphamide-induced gonadotoxicity: subsequent assessment in the adult.

Cyclophosphamide dosage	Other drugs co-administered	Treatment length	Patients within the study		Effect		Reference
			n	Age, years	Short term (1–5 years)	Long term (6–10 years)	
Grams per body weight							
0.2–0.5 g/kg/day	Unknown	1.5–6 months	23 (prepubertal: 16; CYC treated: 23)	3.5–20	Abnormal histology 20–25% of tubules atrophic high cumulative doses	N/A	Pennisi *et al.* (1975)
0.104–0.2 g/kg	Prednisone	49–60 days	4 (CYC treated: 4)	9–13	N/A	Normal testis morphology	
0.312–1.325 g/kg	N/A	89–489 days	4 (CYC treated: 4)	5–8.5	N/A	Damage to seminiferous epithelium with SCOs	Etteldorf *et al.* (1976)
0.475–0.846 g/kg	Asparaginase Cytarabine Doxorubicin Mercaptopurine Methotrexate Prednisolone Vincristine	2–6 years	46	0.08–13	Tubular damage occasionally observed 4 years after treatment with unknown 'low' doses	N/A	Ise *et al.* (1986)
Grams/area							
2.6–29 g/m^2	Asparaginase Cytarabine Dactinomycin Daunomycin Doxorubicin Fluorouracil Mechlorethamine Mercaptopurine Procarbazine Vincristine	Unknown	30 (prepubertal: 19; CYC treated: 10)	1.75–17	N/A	Abnormal morphology	Aubier *et al.* (1989)
2.75–7.5 g/m^2	Asparaginase Carmustine Doxorubicin Mercaptoruine Methotrexate Prednisone Thioguanine Vincristine	3–4 years	17 (prepubertal: 17; CYC treated: 5)	2.5–12.4	Reduced number of GC mm^3	N/A	Müller *et al.* (1988)
Total dose							
0.021–39 g	Fluoxymesterone Mercaptopurine Oxandrolone Prednisone	9 weeks to 19 months	7 (CYC treated: 7)	11–16	High doses; SCOs, peritubular fibrosis, normal morphology LCs. Low doses; active spermatogenesis 90% of tubules	N/A	Penso *et al.* (1974)

Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient's sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.
CYC, cyclophosphamide; GC, germ cell; LC, Leydig cell; SCO, Sertoli cell-only tubule.

primary spermatocytes have also been observed *in vivo* immediately following cisplatin treatment during early puberty (pnd 30–45) in the rat and after a 45-day recovery period (Lirdi *et al*. 2008, Favareto *et al*. 2011). The length of the treatment rather than the cumulative doses appears to be involved in determining the degree of testicular damage (Velez de la Calle *et al*. 1989). Shorter treatment regimens reduce time of exposure of the testis to the chemotherapeutic agent, which could be more important than overall cumulative dose received.

Anthracyclines Of the anthracycline class of drugs, work to date has focused on doxorubicin, and it is clear that this drug targets the pre-mitotic dividing spermatogonia and the pre-meiotic primary spermatocytes in the prepubertal testis (Table 4). These germ cells are undergoing DNA synthesis and therefore contain high levels of the enzyme topoisomerase II, which is a target of the drug (Parvinen & Parvinen 1978). During early prepubertal development, the testis is especially vulnerable to doxorubicin-induced damage, depleting the seminiferous epithelium in comparison to later stages shown in the rat model (Bechter *et al*. 1987). This study found using an immature *in vivo* rat model, that a relatively low dose of doxorubicin (3 mg/kg) was not sufficient to kill all the SSCs, as some recovery was apparent. In addition to inhibition of topoisomerase II activity, doxorubicin has been shown to induce DNA damage in spermatogonia *in vitro*, which can result in cell death (Beaud *et al*. 2017). Interestingly, the Beaud *et al*. (2017) work indicated that cell death was induced independent of apoptosis, since externalization of phosphotidylerine was not apparent following treatment. In agreement with these findings, DNA damage was observed despite the absence of a significant increase in CC3 expression prior to loss of germ cells following short-term exposure of prepubertal mouse testis tissue to doxorubicin (Smart *et al*. 2018). The cell death that occurs following doxorubicin may result from a non-apoptotic mechanism for example through necrosis or autophagy (Beaud *et al*. 2017, Smart *et al*. 2018). This hypothesis would be consistent with studies analysing cardiotoxicity following doxorubicin treatment where the cell death induced is through autophagy (Dirks-Naylor 2013). However, increased levels of CC8 and p53 48 h after treatment in an *in vivo* model has also been reported (Hou *et al*. 2005). Oxidative stress has also been proposed as a mechanism of doxorubicin-induced damage to germ cells, however, work in an *in vitro* model culturing a cell line with rat SSC/spermatogonia type A characteristics (GC-6spg) has shown no such increase in levels of reactive oxygen species (ROS) before the onset of cytotoxicity (Tremblay & Delbes 2018).

Topoisomerase inhibitors Topoisomerase inhibitors, such as etoposide and irinotecan, have been investigated to determine prepubertal gonadal toxicity and have been found to target the pre-mitotic and pre-meiotic germ cells (Table 4). These drugs inhibit the activity of the enzyme topoisomerase I and II and ultimately induce cell death through activation of apoptotic pathways (Freitas *et al*. 2002, Stumpp *et al*. 2004, Ortiz *et al*. 2009). Etoposide damages the prepubertal testis depleting the germ cell pool, with little recovery from treatment at low doses (2 mg/kg; Freitas *et al*. 2002). SSCs are particularly vulnerable to etoposide treatment, with a lower half maximal inhibitory dose in comparison to cisplatin and bleomycin. With etoposide targeting the SSC population, this reduction could account for the reduction in later stages of spermatogenesis observed in the adult rat (Stumpp *et al*. 2004, Marcon *et al*. 2010). Exposure of the prepubertal mouse testis *in vitro* to concentrations of SN38 (the metabolite of irinotecan) that reflect patient serum levels shows that this drug targets the proliferating germ cell population (Lopes *et al*. 2016). Involvement of the apoptotic pathway in cell death has been observed following etoposide treatment in an *in vivo* prepubertal rat model, with increased numbers of apoptotic intermediary and type B spermatogonia, as well as primary spermatocytes, immediately following treatment (Stumpp *et al*. 2004). In addition, increased activity of CC9, CC3 and CC8 as well as enhanced levels of protein and mRNA of p53 and Bcl-2 have been observed in the prepubertal rat following etoposide treatment (Ortiz *et al*. 2009). However, from this study it is not possible to determine which cell types within the testis the observed changes occurred in, since results were obtained from homogenised tissue.

Vinca alkaloids and non-anthracycline antibiotics *In vitro* studies indicate that vincristine may target the germ cell population and bleomycin has the potential to damage the testis (Table 4). Vincristine reduces cell viability and increases cell death of the GC-6spg cell line in a dose- and time-dependent manner. However, this was not a result of DNA damage, as this class of drugs inhibits polymerization of microtubules, involving activation of apoptotic pathways (Beaud *et al*. 2017). Bleomycin targets SSCs *in vitro* in a dose-dependent fashion, with cytotoxicity (seen at 0.1 µM) inducing DNA damage that extends into telomere regions of chromosomes (Marcon *et al*. 2010, Liu *et al*. 2014). These initial studies indicate that such drugs have the potential to impact the testis negatively and should be further investigated *in vivo*.

Combination treatments In a clinical setting, cancer is treated with a combination of chemotherapeutic agents. Different classes of drugs target cells through differing mechanisms which may have synergistic effects. The combined treatments reduce the chance of resistance and survival of cancerous cells, but have the possibility

Table 4 Chemotherapy-induced gonadal toxicity – animal studies.

Chemotherapy drug	**Doses** (length of treatment)	**Animal model** (age)	***In vivo* or *in vitro***	Effect		Reference
				Germ cells	Somatic cells	
(A) Alkylating and alkylating-like agents						
Cisplatin	10–15 µg/mL	Mouse SSCs (isolated from pnd 3 to 6)	*In vitro*	↓ Survival of SSC clusters in dose-dependent manner	N/A	Shabani *et al.* (2016)
	0.1, 0.5 and 1 µg/mL (24 h)	Prepubertal mouse (pnd 5)	*In vitro*	↓ GCs, particularly SSCs ↑ CC3 expression 24 h after treatment ↓ GCs 8 h later ↑ γH2AX GC expression 16 h after treatment	No significant effect on SC or LC numbers	Smart *et al.* (2018)
	1 µM (48 h)	Rat SSCs (isolated from pnd 7 to 8)	*In vitro*	Dose-dependent ↑ γH2AX	N/A	Marcon *et al.* (2010)
	0.5 µg/mL (24 h)	SCs prepubertal rat (pnd 18)	*In vitro*	N/A	50% ↓ transferrin production	Nambu *et al.* (1995)
	200 or 400 µM (24 h)	SCs prepubertal rat (pnd 19)	*In vitro*	N/A	↓ Cell viability (200 µM) but no ↑ CC3 activation at 24 h	Aslani *et al.* (2017)
	0.005 g/kg	Pubertal rat (pnd 30)	*In vivo*	↓ Seminiferous epithelium ↑ Apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes 12 h after treatment	N/A	Lirdi *et al.* (2008)
	0.001 g/kg/day (3 weeks)	Pubertal rat (pnd 45)	*In vivo*	↑ Apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes	Vacuolation of SC ↓ ABP production	Favareto *et al.* (2011)
	0.1 µM (48 h)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑ γH2AX telomeres ↓ Telomere length and activity of telomerase	N/A	Liu *et al.* (2014)
Cyclophosphamide	0.04–0.28 g/kg (2 weeks)	Prepubertal rat (pnd 10)	*In vivo*	No evidence of testicular damage	N/A	Velez de la Calle *et al.* (1989)
	0.002 g/kg/day (5 weeks) 0.7 g/kg total	Pubertal rat (pnd 45)	*In vivo*	Atrophied seminiferous tubules ↓ Spermatogonia and primary spermatocytes	Vacuolation of SC ↓ ABP production	
	0.1 g/kg (10 days)	Pubertal rat (pnd 45)	*In vivo*	No evidence of testicular damage	N/A	
Cyclophosphamide (4-hydroperoxy cyclophosphamide-4OOH-CPA metabolite)	0.1 µM (48 h)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑ γH2AX telomeres expression ↓ Telomere length and activity reduced	N/A	Liu *et al.* (2014)
Cyclophosphamide (arcolein metabolite)	50 or 100 µM (3 or 12 h)	Mouse SCs (isolated from pnd 8)	*In vitro*	N/A	↓ Viability of SC ↑ ROS and ↓ antioxidant activity ↑ Apoptosis Impairs cytoskeleton of SCs	Liu *et al.* (2012)

Chemotherapy drug	**Doses** (length of treatment)	**Animal model** (age)	***In vivo* or *in vitro***	**Effect**		**Reference**
				Germ cells	Somatic cells	
Cyclophosphamide (phosphoramide mustard metabolite)	0.02, 0.2 and 2 µg/mL (24 h)	Prepubertal mouse (pnd 5)	*In vitro*	↓ GCs, particularly SSCs ↑ CC3 expression 16 h after treatment ↓ GCs 8 h later ↑ γH2AX GC expression 16 h after treatment	No effect on SC or LC numbers	Smart *et al.* (2018)
Procarbazine	0.030 g/kg/day (5 or 9 weeks)	Prepubertal (pnd 10), pubertal (pnd 45) and adult rat (pnd 70–90)	*In vivo*	5 weeks; ↓ diameter of SCO (prepubertal), less of an effect on pubertal and adults 9 weeks; less impact on pubertal rats	Vacuolation of SC ↓ ABP production	Velez de la Calle *et al.* (1988)
(B) Anthracyclines						
Doxorubicin	40 and 100 ng/mL (up to 72 h)	Prepubertal rat (pnd 5)	*In vitro*	↓ GC numbers 24 and 48 h ↓ Proliferating and ↑ apoptotic GCs, after 16 h	No effect SCs number, production of key proteins No impact on PMC proliferation or LC testosterone production	Nurmio *et al.* (2009*b*)
	0.05, 0.1 and 0.5 µg/mL (24 h)	Prepubertal mouse (pnd 5)	*In vitro*	↓ GCs, particularly SSCs No expression of CC3 observed before GC loss ↑ γH2AX GC expression 16 h after treatment	No effect on SC or LC numbers	Smart *et al.* (2018)
	0.003 g/kg	Prepubertal (pnd 6 and 16) and pubertal rat (pnd 24 and 45)	*In vivo*	Prepubertal rats ↑ SSC death ↓Toxicity in pubertal rats treated 3, 7 and 14 days after treatment	N/A	Bechter *et al.* (1987)
	0.003 g/kg	Prepubertal rat (pnd 6, 16 and 24)	*In vivo*	Targets migrating gonocytes ↑ Apoptotic cells in pnd 6 rats 48 h after treatment ↑ p53 and CC8 expression	N/A	Hou *et al.* (2005)
	0.4 µg/mL (24 h)	SCs prepubertal rat (pnd 18)	*In vitro*	N/A	35% ↓ transferrin production	Nambu *et al.* (1995)
	0.005 g/kg	Pubertal rat (pnd 22)	*In vivo*	N/A	Dysfunction and morphological alterations pnd 40, more advanced pnd 64 with recovery pnd 127 (adult) ↓ Transferrin production and abnormal positioning of SC nuclei	Brilhante *et al.* (2012)
	0.1 µM (24–72 h)	GC-6 spermatogonial cell line (rat)	*In vitro*	DNA strand breaks Cell death without activation of apoptosis (externalization of phosphatidylserine)	N/A	Beaud *et al.* (2017)
	0.01–10 µM (24 h)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Time- and dose-dependent ↑ in cytotoxicity	Time- and dose-dependent ↑ in cytotoxicity (Ser-W3 more sensitive) ↑ Oxidative stress, nuclear 8-oxo-deooxyguanosine ↓ glutathione levels 6 h Glutathione supplementation did not affect survival	Tremblay and Delbes (2018)

(*Continued*)

Table 4 Continued.

Chemotherapy drug	**Doses** (length of treatment)	**Animal model** (age)	***In vivo* or *in vitro***	**Effect**		**Reference**
				Germ cells	Somatic cells	
Etoposide	1 µM (48 h)	Rat SSCs (isolated from pnd 7 to 8)	*In vitro*	↓ SSC clusters	N/A	Marcon *et al.* 2010
	25 µM or 100 µM (24 h)	SCs from prepubertal rat (pnd 19)	*In vitro*	N/A	No impact on cell viability or CC3 activation.	Aslani *et al.* 2017
	1.2 µg	Prepubertal rat (pnd 21)	*In vivo*	↑ CC9, 8 and 3 activation 24 h after treatment in spermatocytes Protein and mRNA of p53 and Bcl2 altered	N/A	Ortiz *et al.* (2009)
	0.05 g/kg/day Total doses 0.01, 0.02 and 0.04 g/kg	Prepubertal rat (pnd 25)	*In vivo*	Analysed pnd 26 and pnd 32 ↑ Apoptotic differentiated spermatogonia and primary spermatocytes except for 0.01 g/kg group when sacrificed 12 h after end of treatment	N/A	Stumpp *et al.* (2004)
	0.04 g/kg	Prepubertal rat (pnd 25)	*In vivo*	N/A	SC adluminal with chromatin clumps and vacuolization ↓ Transferrin production from pnd 45 onwards	Stumpp *et al.* (2006, 2008)
	0.002 g/kg (30 days)	Pubertal rat (pnd 30)	*In vivo*	↓ GCs ↑ Cell death (chromatin condensation) Damage still apparent 113 days after treatment	N/A	Freitas *et al.* (2002)
	0.01 µM (48 h)	C18-4 spermatogonial cells (mouse)	*In vitro*	No effect on levels of γH2AX levels in telomeres or telomere dysfunction	N/A	Liu *et al.* (2014)
Irinotecan (SN38 metabolite)	0.1 and 1 µg/mL (24 h)	Prepubertal rat (pnd 5)	*In vitro*	Targets the proliferating germ cell population	N/A	Lopes *et al.* (2016)
(D) Vinca alkaloids and antibiotics						
Bleomycin	0.1 µM (48 h)	Rat SSCs (isolated from pnd 7 to 8)	*In vitro*	↓ Cluster number and area in culture	N/A	Marcon *et al.* (2010)
	1.5 µM (48 h)	C18-4 spermatogonial cells (mouse)	*In vitro*	↑ DNA damage in the telomeres, no impact on telomerase activity	N/A	Liu *et al.* (2014)
Mitomycin C	500 µg/kg (alternate day for 20 days)	Pubertal rats (pnd 40)	*In vivo*	N/A	↓ Leydig cell nuclear area ↓ 3β-Hydroxysteroid dehydrogenase	Deb *et al.* (1980)
Vincristine	0.1 µM (48 h)	Rat SSCs (isolated from pnd 7 to 8)	*In vitro*	Targets SSC in a dose-dependent manner	N/A	Marcon *et al.* (2010)
	0.5 µg/mL (24 h)	SCs from prepubertal rat (pnd 18)	*In vitro*	N/A	↓ Transferrin production	Nambu *et al.* (1995)
	0.1 µM (24–72 h)	GC-6 spermatogonial cell line (rat)	*In vitro*	↓ Cell viability ↑ Cell death differentiated spermatogonia dose- and time-dependent manner No observable DNA damage but activation of apoptotic pathways	N/A	Beaud *et al.* (2017)

Chemotherapy drug	Doses (length of treatment)	Animal model (age)	*In vivo* or *in vitro*	Effect		Reference
				Germ cells	Somatic cells	
(E) Combination treatments						
Bleomycin, cisplatin and etoposide	0.1 µM (48 h)	Rat SSCs (isolated from pnd 7 to 8)	*In vitro*	Combination had no additional impact on cluster size/area of SSCs	N/A	Marcon *et al.* (2010)
Vincristine and doxorubicin	0.01 µM (24–72 h)	GC-6 spermatogonial cell line (rat)	*In vitro*	Combination ↑ cell death of spermatogonia dose-dependent manner in comparison to individual treatment	N/A	Beaud *et al.* (2017)

Gonadotoxicity determined with different classes either in isolation or combination. Results suggest that chemotherapy-induced damage is dependent on the chemotherapeutic agent, cumulative dose, stage of development and treatment regimen.
ABP, androgen binding protein; CC, cleaved caspase; GC, germ cell; LC, Leydig cell; PMC, peritubular myoid cell; pnd, postnatal day; SC, Sertoli cell; SCO, Sertoli cell-only tubule; SSCs, spermatogonial stem cells.

of leading to multiplicative off-target side effects on healthy cells. Relatively few animal studies have focused on combinations of drugs (Table 4). Using the GC-6spg cell line, Beaud *et al.* (2017) showed that a combination of vincristine and doxorubicin enhanced levels of cell death in a dose-dependent manner in comparison to treatment with each drug individually. In contrast, exposure to a combination of bleomycin, cisplatin and etoposide had no additional impact on cluster size/area of mouse SSCs in culture (Marcon *et al.* 2010). Further investigations into common combinations are required to determine the relative gonadotoxicity, and whether the effects are synergistic and/or multiplicative.

Somatic cell effects

Studies relating to impacts of chemotherapy treatment upon the somatic cells have mainly been limited to the Sertoli cell and include studies which have focused on cyclophosphamide, cisplatin, doxorubicin and etoposide chemotherapy agents (Table 4). Treatment results in morphological damage including vacuolation of the cells and adluminal positioning (Velez de la Calle *et al.* 1988, 1989, Stumpp *et al.* 2006, 2008, Favareto *et al.* 2011, Brilhante *et al.* 2012). However, these cells do survive exposure as shown in Smart *et al.* (2018) where there was no overall change in cell numbers following cyclophosphamide, cisplatin or doxorubicin treatment in an *in vitro* model of prepubertal mouse testicular tissue. Damage to the Sertoli cells resulting in dysfunction could be the primary effect of such treatment, which would have a significant impact on testis function. Indeed, decreased production of androgen-binding protein following treatment with alkylating and alkylating-like agents has been reported in prepubertal rats (Velez de la Calle *et al.* 1988, 1989, Favareto *et al.* 2011). *In vitro* studies using primary cultures of rat Sertoli cells have also shown reduced transferrin production following cisplatin, doxorubicin and vincristine treatment (Nambu *et al.* 1995). Transferrin stimulates germ cell proliferation/differentiation by transferring iron to these cells (Sylvester & Griswold 1994). A potential mechanism by which cyclophosphamide and doxorubicin induce damage specifically to the Sertoli cell has been proposed by *in vitro* studies of cultured immature Sertoli cells where enhanced levels of oxidative stress have been reported (Liu *et al.* 2012, Tremblay & Delbes 2018) as well as damage to the cytoskeleton following cyclophosphamide treatment (Liu *et al.* 2012).

Impairment of Sertoli cell functionality, however, may be secondary and a result of primary injury to the germ cell population. An *in vivo* study looking at doxorubicin-induced damage has shown that the alterations in the morphology and function of these cells was more pronounced in early adulthood in comparison to time of treatment just before puberty (pnd 22). This suggests that the germ cells were the primary cells targeted

method clinically (reviewed Giudice *et al.* 2017). Xenotransplantation of prepubertal human testicular tissue for up to 9 months has been performed with nude mice where initiation of spermatogenesis was observed within the grafted tissue, however spermatogenesis did not reach meiotic differentiation (Wyns *et al.* 2007, 2008, Goossens *et al.* 2008, Van Saen *et al.* 2011, 2013). SSCs isolated from prepubertal patients have been propagated *in vitro*, but results are preliminary (Sadri-Arekani *et al.* 2011). Human prepubertal testicular tissue has been successfully cultured *in vitro* with survival of the spermatogonial germ cell population, maturation of the somatic cells and formation of the blood–testis barrier reported (de Michele *et al.* 2017, 2018). Initiation of spermatogenesis from such tissue has yet to be demonstrated. Alternatively, the prepubertal testis could be protected against injury caused by the treatment through use of cytoprotective agents that would be added to the cancer treatment regimen. Several studies have shown the gonadoprotective potential of agents in animal studies focusing on adult males, mainly investigating the morphology and motility of sperm. For example, Carmely *et al.* (2009) has shown in a mouse model the cytoprotective effect of the immunomodulator AS101 compound against cyclophosphamide-induced testicular damage. In contrast, so far only a limited number of compounds have been analysed to determine their ability to protect the prepubertal testis in an animal model (Table 5).

Amifostine

Amifostine is an organic thiophosphate that acts as a cytoprotective agent, protecting cells against chemotherapeutic damage whilst having no antitumor activity (Çetingül *et al.* 2009). The active metabolite acts as a ROS scavenger and binds and stabilizes DNA (Spencer & Goa 1995). This drug has a limited half-life of eight minutes, therefore *in vivo* studies have focused on pre-treatment of amifostine 15 min before chemotherapy treatment. Pre-treatment partially protected the prepubertal rat testis against cisplatin- and doxorubicin-induced testicular damage; this effect may be dependent on the age and schedule of treatment, as a lower dose of amifostine in earlier stages of prepubertal development in rats had no protective effects against doxorubicin-induced damage when tissues were analysed 24 and 48 h after treatment (Jahnukainen *et al.* 2001, Hou *et al.* 2005). However, prepubertal amifostine pre-treatment before doxorubicin treatment did not maintain fertility in the adult, as DNA damage was found in the sperm of treated animals when analysed 64 days after treatment, which increased the number of arrested embryos in a mating study of adults (100 days old) (Vendramini *et al.* 2012). An *in vitro* study has also indicated that amifostine has no protective effects against doxorubicin-induced damage in a spermatogonial cell line and an immature Sertoli cell line (Tremblay & Delbes 2018). Therefore, amifostine may not be a suitable fertility preservation strategy.

Carnitine

Carnitine is a quaternary amine found at high concentrations within the epididymis of the male reproductive tract and in spermatozoa. It is acquired through dietary meat and milk and is also produced by the liver through methylation of lysine and methionine amino acids. This compound has essential roles in determining male fertility, producing energy by transferring long-chain fatty acids into mitochondria, a process required for germ cell maturation, sperm motility and sperm count; it has been shown to have cytoprotective properties whilst having no impact on anticancer treatment efficacy (Chiu *et al.* 2004, Sayed-Ahmed 2010). Partial protection from etoposide and doxorubicin-induced morphological damage and apoptotic germ cell death was shown when prepubertal rats were pre-treated with carnitine one hour before chemotherapy treatment *in vivo* (Okada *et al.* 2009, Cabral *et al.* 2014). The mechanism underlying such cytoprotective action is unknown, but may be the result of enhanced DNA repair activity, inhibition of ceramide production and/or reduction in oxidative stress-induced damage as shown in a range of different cell types (Andrieu-Abadie *et al.* 1999, Palmero *et al.* 2000, Alshabanah *et al.* 2010). Indeed, a study by Cabral *et al.* (2018) has shown improved oxidative stress status of the adult testis following prepubertal pre-treatment with cartinine one hour before doxorubicin treatment in a rodent model. An *in vitro* model, however, has found no protective effects against doxorubicin-induced damage when cartinine is administered in spermatogonial or immature Sertoli cell lines (Tremblay & Delbes 2018). Sertoli cell function may be improved upon carnitine administration, as these cells express carnitine/organic cation transport 2 receptors, with carnitine important in the maintenance of the BTB (Palmero *et al.* 2000).

Ginseng intestinal metabolite I (GIM-I)

The herbal root, Ginseng has been used in East Asian countries as a traditional Chinese medicine and the intestinal metabolite, known as ginseng intestinal metabolite I (GIM-I), is thought to have multiple pharmacological effects through its antioxidant activity (Zhang *et al.* 1996). The protective effect against doxorubicin-induced damage has been analysed by Kang *et al.* (2002) in prepubertal mice *in vivo*, where GIM-I was found to partially protect against doxorubicin-induced germ cell damage resulting in testicular morphology comparable to controls that may be the result of enhanced antioxidant activity. GIM-I

by doxorubicin, with loss/damage to the germ cells ultimately impacting Sertoli cells as a secondary effect (Brilhante *et al*. 2012). For etoposide, functional deficits were also more apparent in adulthood, suggesting damage was secondary to germ cell death. However, upon recovery of the seminiferous epithelium, Sertoli cell dysfunction was still apparent with reduced transferrin production and altered morphology; this may suggest a degree of primary damage on the Sertoli cells (Stumpp *et al*. 2006). How to distinguish between primary and secondary damage to the Sertoli cells is difficult due to the dependence of Sertoli cells on germ cells and vice versa. Isolation of Sertoli cells from the germ cell population, as with the primary cell culture or established cell line culture experiments, has been used to look specifically at Sertoli cell damage. These isolated Sertoli cells, however, may not be representative of the 'true' *in vivo* Sertoli cell as often these cells do not maintain Sertoli cell identity once removed from their true environment, a limitation that needs to be borne in mind for all cell line studies.

To date, few studies have focused on the other somatic cell types within testis, the Leydig cells or peritubular myoid cells. An *in vivo* study reported reduced steroidogenic activity of Leydig cells following treatment of immature rats with mitomycin C (Deb *et al*. 1980). More recent *in vitro* studies have shown there was no change in Leydig cells numbers reported by Smart *et al*. (2018) after *in vitro* exposure of mouse prepubertal testis fragments to cisplatin, cyclophosphamide and doxorubicin. The proliferative ability of the peritubular myoid cells and steroidogenic activity of the Leydig cells were also unaltered after doxorubicin treatment of rat testis tissue *in vitro* (Nurmio *et al*. 2009*b*). Additional studies looking specifically at these cell types within the testis are required before any conclusion can be drawn on chemotherapy-induced damage.

Summary of animal studies

To conclude, in the prepubertal testes chemotherapy agents have been shown to specifically target and deplete the germ cell pool, in some cases specifically the SSC population, with DNA damage noted after cyclophosphamide, cisplatin and doxorubicin exposure. Apoptosis is the main cell death pathway activated by cyclophosphamide, cisplatin, etoposide and vincristine exposure, whereas doxorubicin-induced testicular damage may be the result of an alternative cell death pathway such as necrosis or autophagy. Cancer therapy may also affect the Sertoli cell population resulting in morphological damage and/or dysfunction, as shown by cyclophosphamide, procarbazine, cisplatin, doxorubicin and etoposide treatment. However, somatic cell impairment may either be the result of primary cellular damage to the Sertoli cells themselves and/or a secondary consequence of targeted loss of the germ cell population. Whether combined chemotherapy treatment regimens modify the toxicity of individual drugs needs further clarification, as few such *in vitro* studies have been performed to date.

Effects on future generations

The clinical impact of prepubertal chemotherapy treatment on later fertility has been discussed in several reviews (Hudson 2010, Lee & Shin 2013); for survivors who are able to conceive there could be potential effects upon future generations due to unrepaired damage to the male germline. The impact on future generations is not yet clear. A Danish study of 472 survivors of childhood cancer found no significant association between alkylating chemotherapy treatment and later genetic diseases of the progeny of these survivors (Winther *et al*. 2012). Nonetheless, a study by Liu *et al*. (2014) has shown a potential mechanism by which alkylating agents can impact on future generations by targeting the telomeres of mouse spermatogonial cells. Telomerase function was reduced at concentrations of a drug precursor of cyclophosphamide (4OOH-CPA) and cisplatin which induced significant spermatogonial cell death resulting in reduced telomere length and activity of telomerase. A reduction in the length or function of telomeres of the male germ cell can adversely affect early development of the offspring, increasing the rate of pre- and/or post-implantation loss, congenital malformation and miscarriage (Liu *et al*. 2002). Whether additional classes of drugs also impact on future generations is unknown and therefore further research into this area is urgently required.

Protective strategies

The development of chemotherapeutic treatment regimens has increased greatly in the past decade resulting in a greater number of childhood cancer survivors reaching adulthood and facing long-term consequences of treatment, such as infertility. There is therefore an increasing focus on preserving the fertility of children undergoing cancer treatment. At the time of writing, though, fertility preservation strategies for prepubertal boys have yet to be established clinically. Testicular tissue from boys undergoing 'high' risk chemotherapy treatment is being collected in a limited number of centres, with tissue cryopreserved and stored for potential fertility restoration when the boys reach adulthood. The proposed techniques to restore fertility, however, are still in the experimental phase of development with success to date only in rodent models (Picton *et al*. 2015, Giudice *et al*. 2017). Studies with human tissues have been conducted in relation to transplantation of cryopreserved testicular tissue and SSCs as well as *in vitro* maturation of immature testicular tissue, paving the way for the development of a fertility restoration

Table 5 Potential cytoprotective agents to protect the prepubertal testis against chemotherapy-induced damage.

Compound	Dose	Chemotherapy drug/doses	Animal model/age	*In vivo* or *in vitro*	Effect	Reference
Amifostine	0.2 g/kg	Doxorubicin 0.003 g/kg	Prepubertal rat (pnd 6)	*In vivo*	No protective effects	Jahnukainen *et al.* (2001), Hou *et al.* (2005)
Amifostine	0.4 g/kg	Cisplatin 0.005 g/kg	Prepubertal rat (pnd 30)	*In vivo*	Partial protection, ↓ seminiferous tubule area and ↑ apoptotic spermatogonia and primary spermatocytes	Lirdi *et al.* (2008)
Amifostine	0.4 g/kg	Doxorubicin 0.005 g/kg	Prepubertal rat (pnd 30)	*In vivo*	Partially protects. Did not protect against DNA damage and negatively impacted on embryo development and pregnancy outcome	Vendramini *et al.* (2010, 2012)
Amifostine	1 µM	Doxorubicin 0.01–1 µM (24 h)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 h or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line	Tremblay & Delbes (2018)
Cartinine	0.25 g/kg	Etoposide 0.04 g/kg	Prepubertal rat (pnd25)	*In vivo*	Analysed pnd 30, 64 and 100. Partial protection, reduction in TUNEL+ cells	Okada *et al.* (2009)
Cartinine	0.25 g/kg/day	Doxorubicin 0.005 g/kg	Prepubertal rat (pnd30)	*In vivo*	Analysed pnd 64 and 100. Partial protection, ↓ TUNEL+ cells and sperm DNA damage. ↑ Acrosome integrity pre-treatment, no impact on sperm motility and mitochondrial activity. ↓ Lipid peroxidation and nitric oxide. ↑ Fertility index and implantation rate improved	Cabral *et al.* (2014, 2018)
Cartinine	10 mM	Doxorubicin 0.01–1 µM (24 h)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 h or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line	Tremblay & Delbes (2018)
Curcumin	5 µM	Doxorubicin 0.01–1 µM (24 h)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 h or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line	Tremblay & Delbes (2018)
Ginseng intestinal metabolite I (GIM-I)	0.05 g/kg/day	Doxorubicin 0.05 g/kg/day	Prepubertal rat (pnd28)	*In vivo*	Partially protects	Kang *et al.* (2002)
Vitamin C	40 µg/mL	Doxorubicin 0.01–1 µM (24 h)	GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)	*In vitro*	Pre-treatment for 24 h or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line	Tremblay & Delbes (2018)

increases the levels of testis-specific antioxidants which were reduced following doxorubicin treatment. This compound has great potential as a cytoprotective agent against doxorubicin-induced damage and appears to have anti-metastatic activity and could therefore be added to chemotherapeutic regimens to provide benefit as both a cytotoxic and cytoprotective agent (Hasegawa *et al.* 1997).

Vitamin C and curcumin

A study by Tremblay and Delbes (2018) has investigated the potential of compounds as cytoprotectants based upon their antioxidant activity, including vitamin C and curcumin. Vitamin C can also function by inhibiting apoptosis, whilst curcumin has additional anti-inflammatory properties. However, in both cases treatment did not reduce the cytotoxic activity of doxorubicin in either spermatogonial or immature Sertoli cell lines. This study therefore indicates that these compounds may not be suitable cytoprotectants against doxorubicin-induced prepubertal testicular damage.

Summary of protective strategies

Cytoprotective agents could play a major role in the future of fertility preservation strategies, however, research in this area to date is very limited, and has only shown partial protection against chemotherapy-induced damage with pre-treatment with amifostine, cartinine and GIM-I. Moreover, prepubertal amifostine treatment alone compromised later fertility and is therefore not suitable for purpose. In contrast, GIM-I might have more potential as a cytoprotective agent, in part due to its anti-metastatic activity. Overall, despite the promising results outlined, the level of evidence established so far in animal studies in not sufficient for transfer to clinical practise and needs further investigation.

Conclusion

This review has provided an overview of what is currently known in relation to chemotherapy-induced prepubertal testicular toxicity from studies in human patients and animal models, focusing primarily upon direct damage following chemotherapy exposure. Cancer therapy with a range of chemotherapy agents from different drug classes during childhood have been found to negatively impact upon the prepubertal testis. The resulting damage depends on the compounds used, cumulative dose, administration regimen and age/pubertal status during treatment. Such conclusions have been drawn from both clinical investigations and animal models, including *in vivo* as well as *in vitro* studies, with testicular fragments or primary cell cultures/cell lines representatives of the cell types in the prepubertal testis. However, as this review has discussed, the evidence for chemotherapy-induced damage to the prepubertal testis is at present incomplete and needs further investigation.

Further research into chemotherapy-induced prepubertal testicular toxicity is essential as the number of childhood cancer survivors is set to increase steadily over the coming years. Enhancing our knowledge of the gonadotoxicity of chemotherapeutic agents is essential for clinicians to determine which patients to offer cryopreservation of immature testicular tissue for potential fertility restoration strategies, which are at present experimental for human patients. Understanding how chemotherapy agents target and damage the testis of young boys will provide much clarity to the future quality of life of these patients and aid in the development of protective strategies for preservation of fertility.

Declaration of interest

Norah Spears is a member of the Editorial Board of *Reproduction*. None of the other authors has any conflict of interest.

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Appendix B (Age-matched controls)

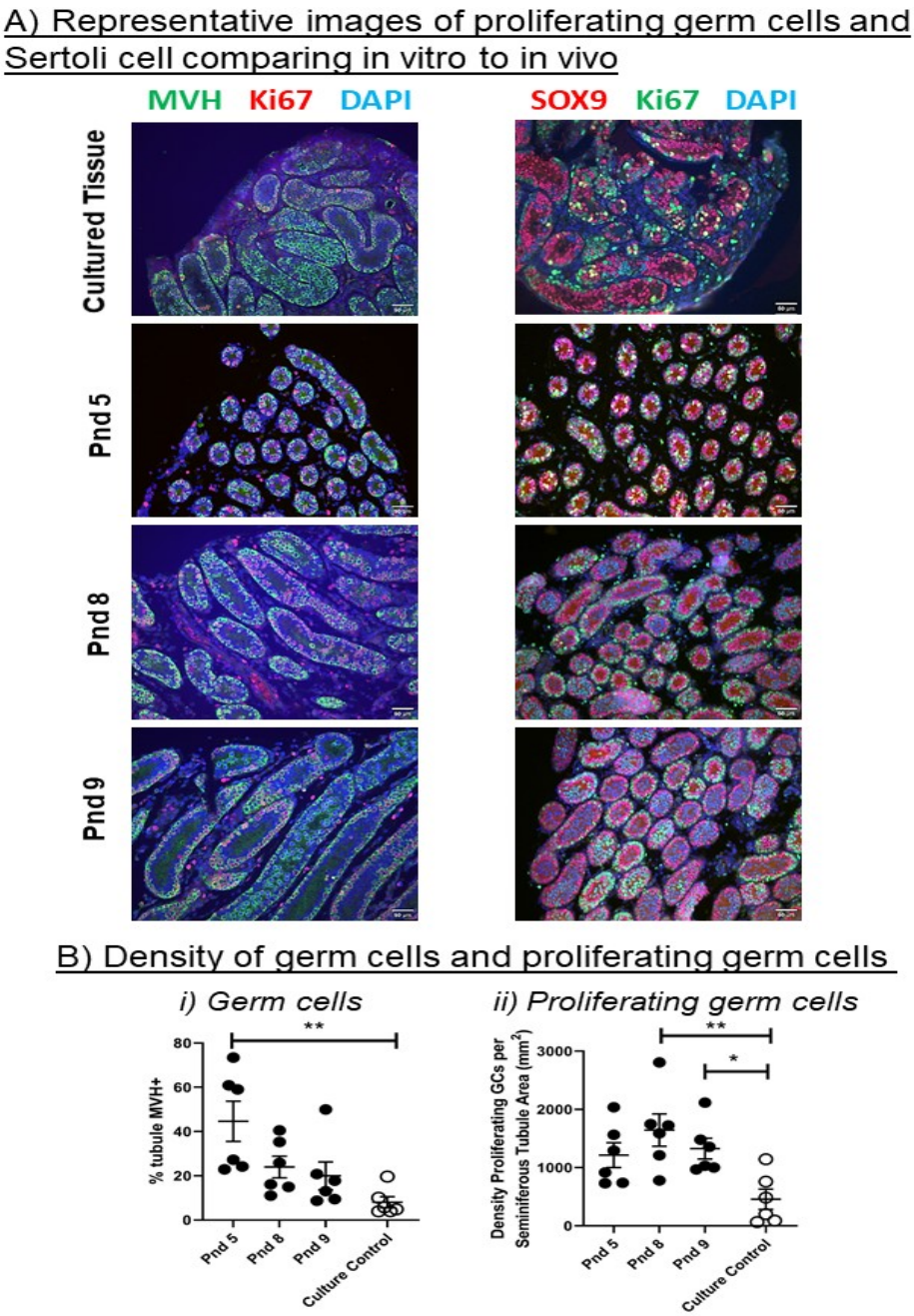
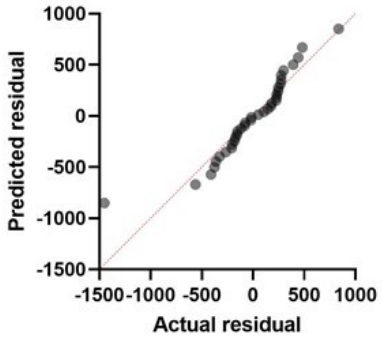


Figure 1: Comparison of *In vitro* testes to *In vivo* age-matched controls (pnd five, start of culture and pnd eight/nine, end of culture). A) Representative images of germ cells and Sertoli cells. Bi) Total germ cell density and Bii) proliferating germ cell density per seminiferous tubule area. Density of germ cells similar to end of culture controls, whereas proliferative capacity of germ cells reduced. Images were taken at 20X for analysis and scale bar = 50µm. 'n'=6 for germ cells, 'n'=4 for Sertoli cells. Data analysed with one-way ANOVA followed by Dunnetts post-hoc test for. *p<0.05, ** p<0.01, comparing age-matched controls to culture control. Data are shown as mean +/- SEM.

Appendix C (QQ plots to determine normal distributions)

i) Total germ cell density

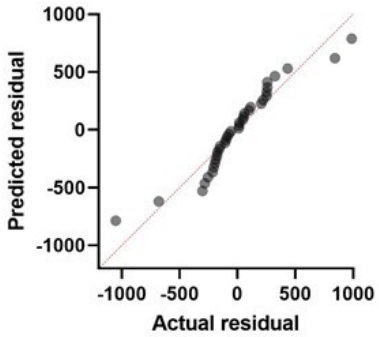
#



p=0.0041

ii) Proliferating germ cell density

#



p=0.0113

iii) Non-proliferating germ cell density

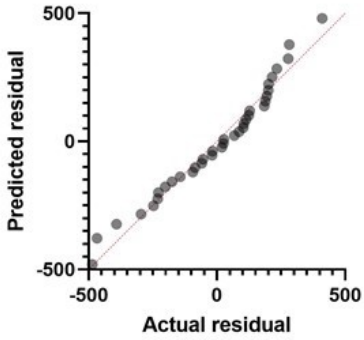


Figure 1: QQ plot of germ cell density following vincristine-induced damage to the prepubertal mouse testis. Data for i) total germ cell density, ii) proliferating germ cell density and iii) non-proliferating germ cell density quantiles graphed against those of a normal distribution. (#) The data for i) and ii) did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ deemed the data did not follow a normal distribution. Therefore, non-parametric tests were used to analyse i) and ii). iii) was deemed to follow a normal distribution and therefore parametric variations of statistical tests were used.

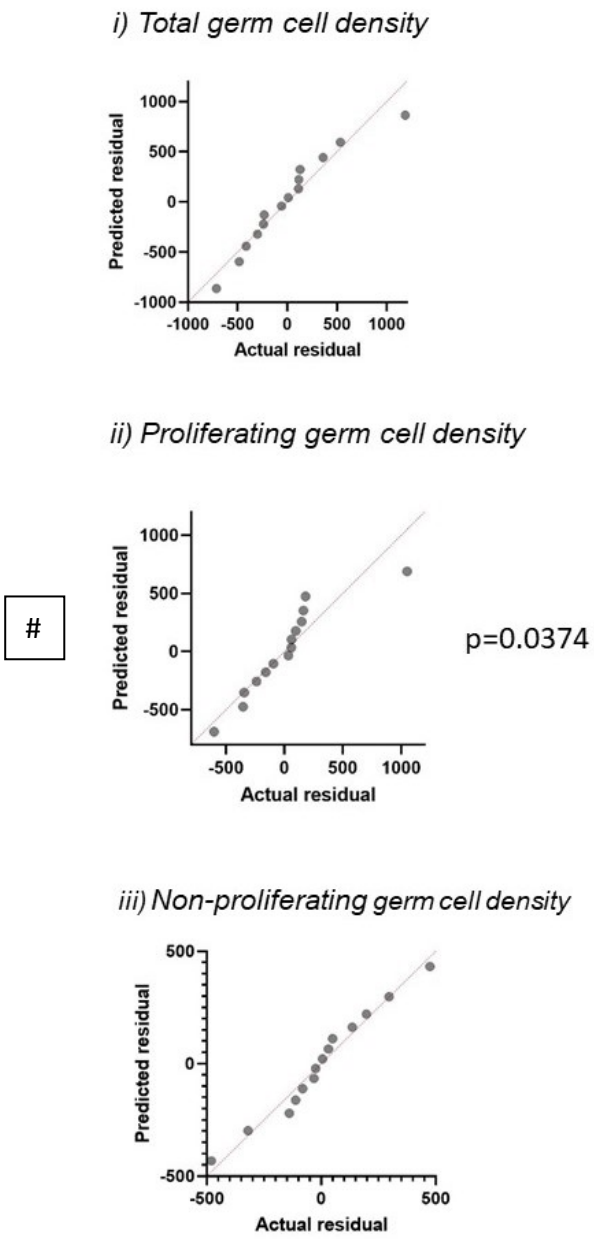
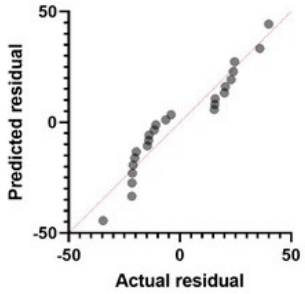


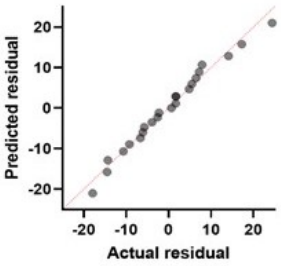
Figure 2: QQ plot of germ cell density following doxorubicin-induced damage to the prepubertal mouse testis. Data for i) total germ cell density, ii) proliferating germ cell density and iii) non-proliferating germ cell density quantiles graphed against those of a normal distribution. (#) Data ii) did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ deemed the data did not follow a normal distribution. Therefore, non-parametric tests were used to analyse ii). i) and iii) were deemed to follow a normal distribution and therefore parametric variations of statistical tests were used.

i) Cisplatin-induced damage



p=0.0233

ii) Doxorubicin-induced damage



iii) Vincristine-induced damage

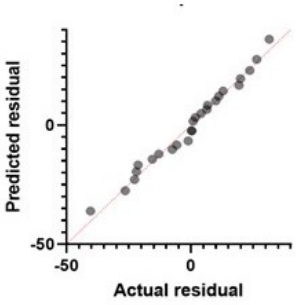


Figure 3: QQ plot of cell viability following chemotherapy-induced damage to GC-6spg cell line. Data for i) cisplatin-induced, ii) doxorubicin-induced and iii) vincristine-induced cytotoxicity quantiles were graphed against those of a normal distribution. (#) Data i) did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ deemed the data did not follow a normal distribution. Therefore, non-parametric tests were used to analyse i). ii) and iii) were deemed to follow a normal distribution and therefore parametric variations of statistical tests were used.

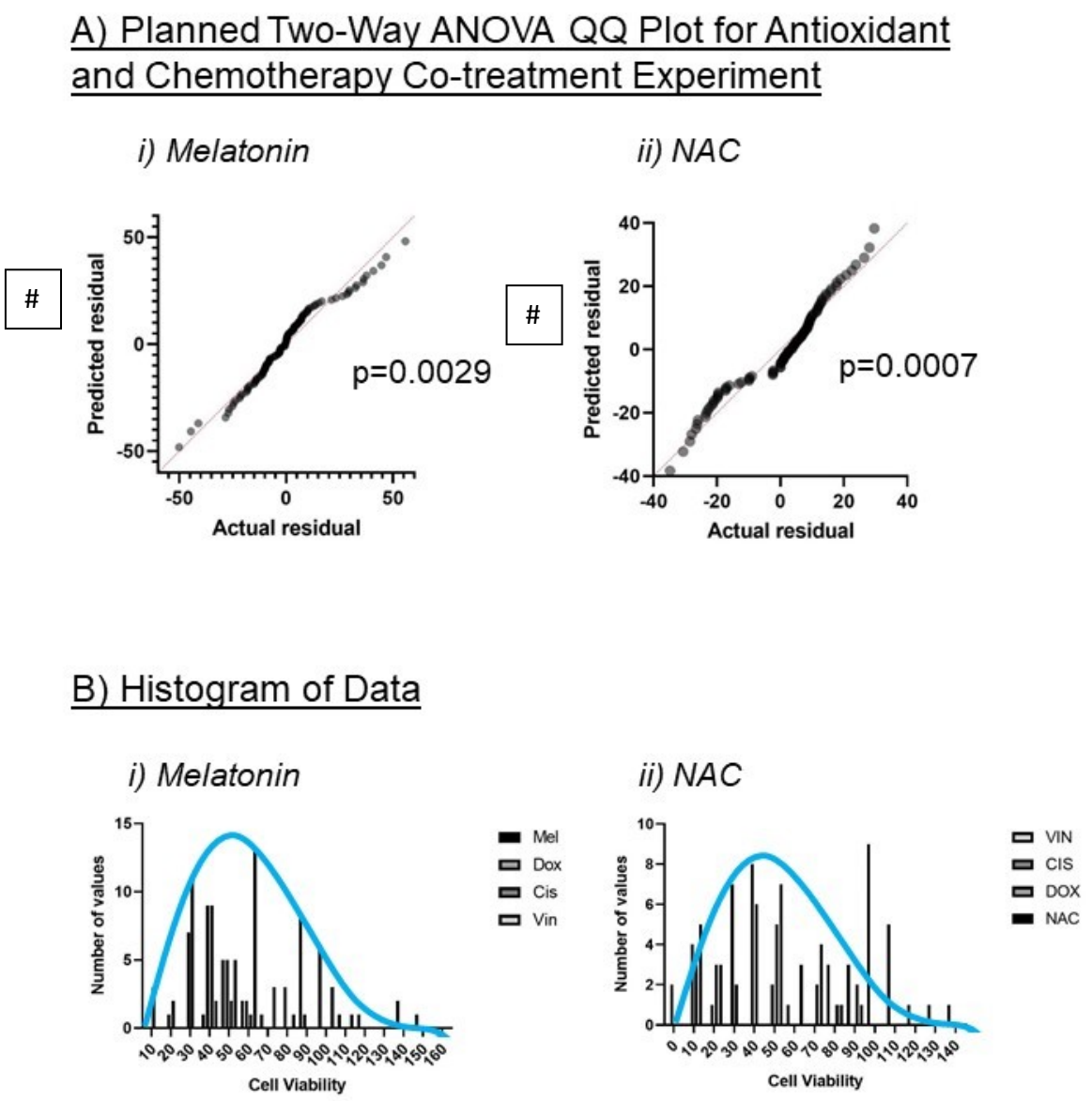


Figure 4: Normal distribution analysis for planned two-way ANOVA analysis of antioxidant-co treatment data. A) QQ plots of i) melatonin co-treatment experiment and ii) NAC co-treatment experiment where data was graphed against those of a normal distribution. (#) Data did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ and deemed the data did not follow a normal distribution. Therefore, non-parametric tests were required but could not be used for planned two-way ANOVA. B) Histogram of data showing left-hand skew.

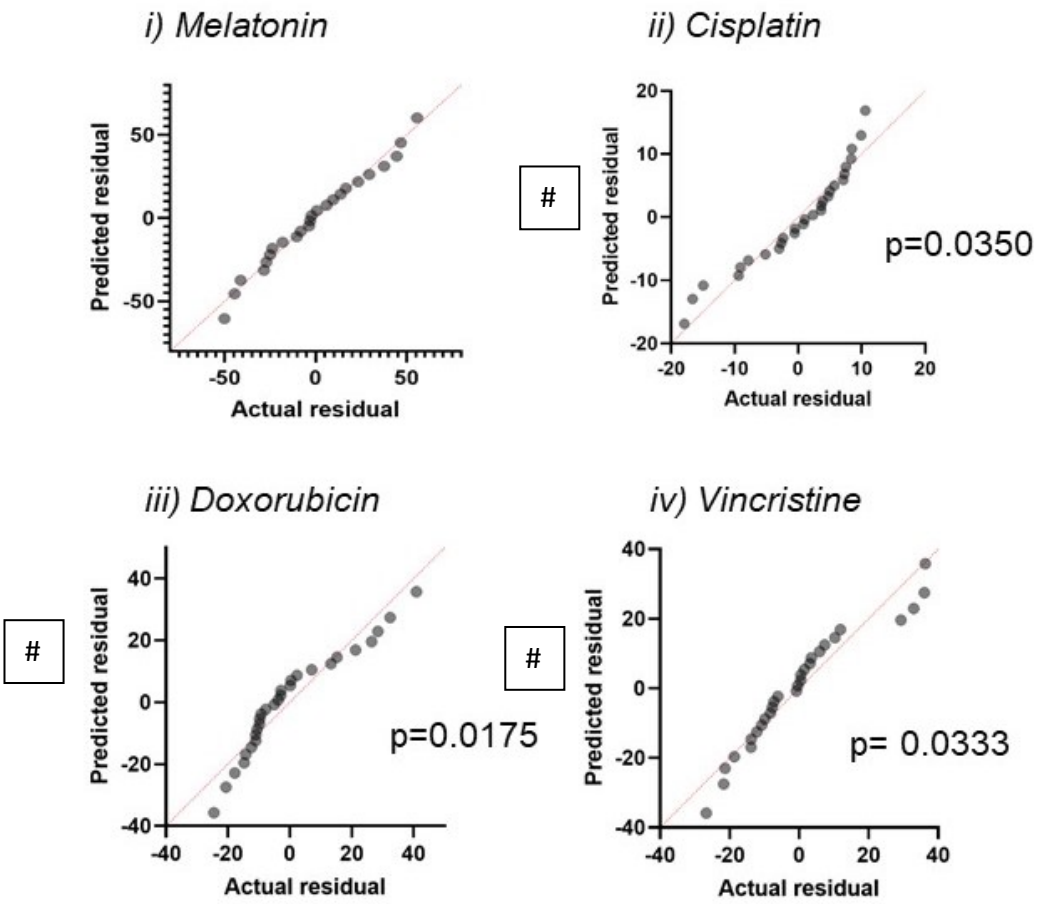


Figure 5: QQ plot of cell viability following co-treatment of chemotherapy and melatonin to GC-6spg cell line. Data for i) melatonin treatment, ii) melatonin and cisplatin co-treatment, iii) melatonin and doxorubicin co-treatment and iv) melatonin and vincristine co-treatment. Quantiles were graphed against those of a normal distribution. (#) Data ii), iii) and iv) did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ deemed the data did not follow a normal distribution. Therefore, non-parametric tests were used to analyse ii), iii) and iv). i) data wa deemed to follow a normal distribution and therefore parametric variations of statistical tests were used.

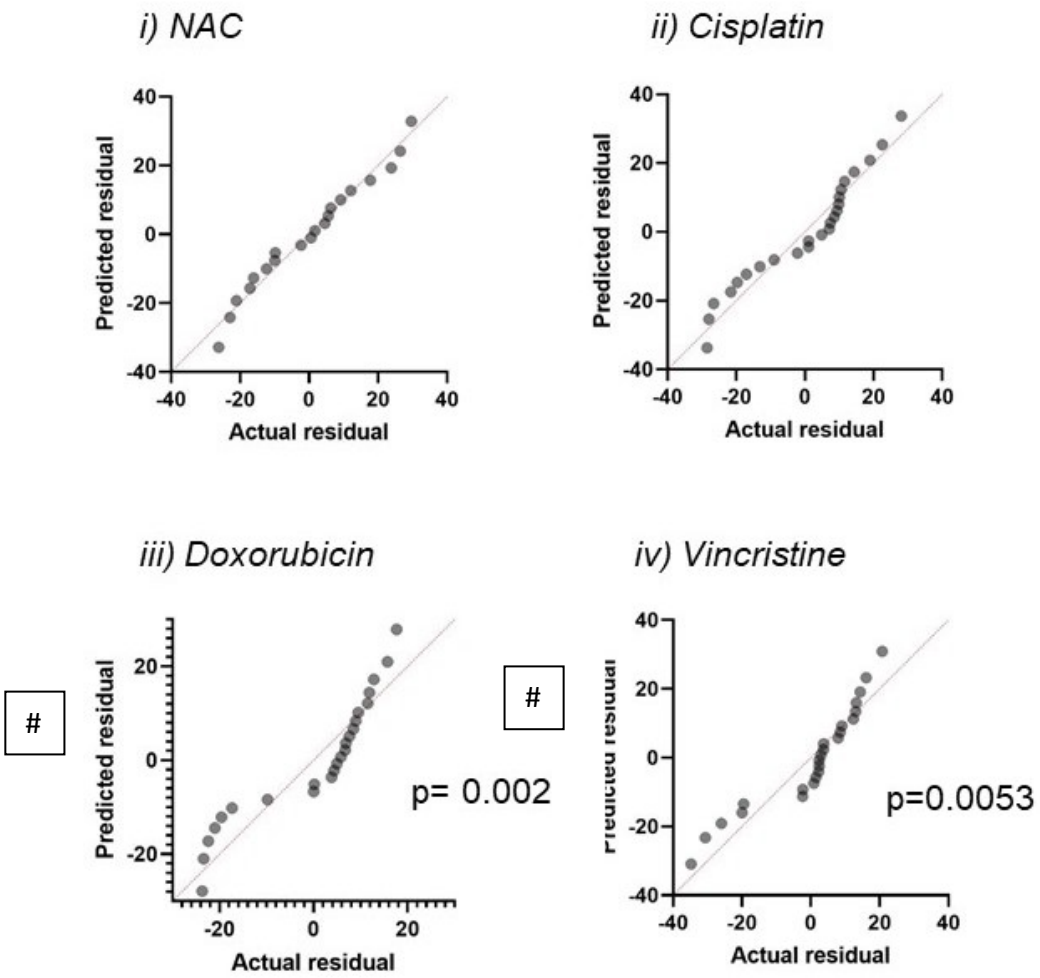


Figure 6: QQ plot of cell viability following co-treatment of chemotherapy and NAC to GC-6spg cell line. Data for i) NAC treatment, ii) NAC and cisplatin co-treatment, iii) NAC and doxorubicin co-treatment and iv) NAC and vincristine co-treatment. Quantiles were graphed against those of a normal distribution. (#) Data ii), iii) and iv) did not appear to follow a normal distribution, therefore Shapiro Wilks test was performed to confirm observations where $p<0.05$ deemed the data did not follow a normal distribution. Therefore, non-parametric tests were used to analyse ii), iii) and iv). i) data was deemed to follow a normal distribution and therefore parametric variations of statistical tests were used.