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The effect of chemotherapy on the prepubertal testis

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Thesis for the degree of Master of Science by Research
in Biomedical Sciences (Integrative Physiology)

University of Edinburgh

2015



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Declaration

I hereby declare that this work has been composed by me and all the work is my own, except where indicated. This work has not been submitted for any other degree or professional qualification except as specified. Any work by other authors has been indicated and any outside assistance has been clearly identified in the **assistance received** chapter of the thesis.

Eleanor Smart

August 24, 2015

Abstract

With an increase in long term survival rates for childhood cancers, more focus is now being placed on quality of life issues resulting from long term side effects of treatment. Infertility in later life is a known side effect of chemotherapy in patients that have survived childhood cancer. The testis contains two irreplaceable cell populations for subsequent fertility, the proliferating stem germ cells and the somatic Sertoli cells that support the development of the germ cells. First different methods to culture mouse testicular tissue were examined and then compared to *in vivo* time points to validate the model used. In order to examine which cell type is effected by exposure to chemotherapy, neonatal mouse testes were exposed to varying concentrations of either phosphoramidate mustard (PM), cisplatin (CIS), or doxorubicin (DOX) on the second day of a four day *in vitro* culture ($n \geq 8$ for each treatment). Immunofluorescent analysis showed that exposure to PM, CIS and DOX resulted in a specific loss of germ cells in the neonatal mouse testis at doses within the range found in patients' serum. Finally, preliminary cultures using human foetal tissue were developed in order to compare the effects of chemotherapy between the mouse and human. In conclusion, the results presented here suggest that infertility following chemotherapy in childhood is due to a specific loss of germ cells from the seminiferous tubules.

Lay summary

Survival rates for paediatric cancers have dramatically increased. The majority of these patients are treated with chemotherapy that has a mix of short and long term side effects. Infertility is a common side effect after chemotherapy. The childhood testis contains germ cells that will divide and produce sperm in later life and specialised support cells that aid in the development of the germ cells. Either of these cell populations could be damaged by chemotherapy and result in infertility in adulthood.

We took pieces of mouse testis and exposed them to three chemotherapeutic agents phosphoramidate mustard, cisplatin and doxorubicin at a range of concentrations that are found within the blood of patients undergoing chemotherapy. A range of markers for different cell types and for cell death were examined. When the tissue was exposed most of the germ cells were lost from the testis without a change in support cell number. The amount of cell death also increased but there was still very little in total.

These results indicate that the three chemotherapy drugs studied here can cause a specific decrease in the germ cells in the neonatal mouse testis. This could result in infertility in later life.

Assistance received

Chapter 2

Dissection

All mouse handling and dissections were performed for me by trained members of the Spears' lab (See 2.1.1).

Histology

Processing of tissue and embedding into paraffin blocks was conducted by Vivian Allison or Louise Dunn (See 2.4).

Chapter 3

Siobhan Rice (undergraduate student) assisted me with the immunohistochemical analysis of the *in vivo* time point analysis (See 3.4.4.2).

Chapter 4

Siobhan Rice (undergraduate student) under my supervision assisted in the cultures, histology and immunohistochemistry and data analysis of the phosphoramidate mustard cultures (See 4.3.1).

Chapter 5

SRY of first trimester human foetal testis samples was performed by Maria Camacho Moll (See 5.3.2).

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List of abbreviations

3β-HSD	3- β -hydroxysteroid dehydrogenase
ALSC	adult Leydig stem cell
AMH	anti-Müllerian hormone
ANOVA	analysis of variance
AR	androgen receptor
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CC3	cleaved caspase-3
CIS	cisplatin; cis-diammineplatinum(II) dichloride
COUP	chicken ovalbumin upstream promoter
COUPTFII	COUP transcription factor 2
CSF-I	colony stimulating factor 1
CYP	cyclophosphamide
DAPI	4',6-diamidino-2-phenylindole
DAZL	deleted in azoospermia-like
DOX	doxorubicin; doxorubicin hydrochloride
DPBS	Dulbecco's phosphate-buffered saline
DPX	distyrene, plasticizer and xylene
e	embryonic day
ESC	embryonic stem cell
FGF2	fibroblast growth factor 2
FLC	foetal Leydig cell
FSH	follicle stimulating hormone
GDNF	glial cell-derived neurotrophic factor
H&E	haematoxylin and eosin

ICSI	intracytoplasmic sperm injection
iPSC	induced pluripotent stem cells
ITS	insulin, transferrin and selenium
KRS	knockout replacement serum
LH	luteinizing hormone
MAGE-A4	melanoma-associated antigen 4
MEM	minimal essential medium
MVH	mouse VASA homologue
NBF	neutral buffered formalin solution
NRES	National Research Ethics Service
OCT4	octamer-binding transcription factor 4
PBS	phosphate-buffered saline
PBSTx	1 × PBS + 0.1% Tritonx
PM	phosphoramidate mustard
pnd	postnatal day
PTM	peritubular myoid cell
SEM	standard error of the mean
SOX9	SRY-box 9
SRY	sex-determining region Y
SSC	spermatogonial stem cell
TBS	tris buffered saline

Chapter I. General introduction

I.1 Embryonic testis development

I.1.1 Testis formation

The mammalian gonad forms from a bi-potential genital ridge that has the ability to differentiate into an ovary or testis (Koopman et al., 1991). The somatic cells and germ cells arise from different locations in the embryo. The support cells of the testis, the Sertoli cells develop from the surface of the coelomic epithelium (Karl and Capel, 1998). It is these differentiating Sertoli cells (pre-Sertoli cells), that initiate testicular development. Primordial germ cells migrate through the gut mesentery into the urogenital ridge to populate the gonad (Anderson et al., 2000), where they are enclosed in cords by the pre-Sertoli cells (Fig. 1.1). Germ cells are not required for cord formation; when they are depleted, seminiferous cords still form (Pellas et al., 1991, Merchant, 1975). Vascular endothelial cells aided by macrophages are also necessary as they migrate from the mesonephros and partition the cords (Fig 1.1; (Combes et al., 2009, DeFalco et al., 2014). Macrophages also aid the clearance of mislocated germ and Sertoli cells that have not made it into cords thus further aiding the partitioning of cords and interstitium (DeFalco et al., 2014). Peritubular myoid cells (PTMs) surround the Sertoli cells and form a basement membrane surrounding the seminiferous cords, separating the interstitial space from the tubules (Skinner et al., 1985).

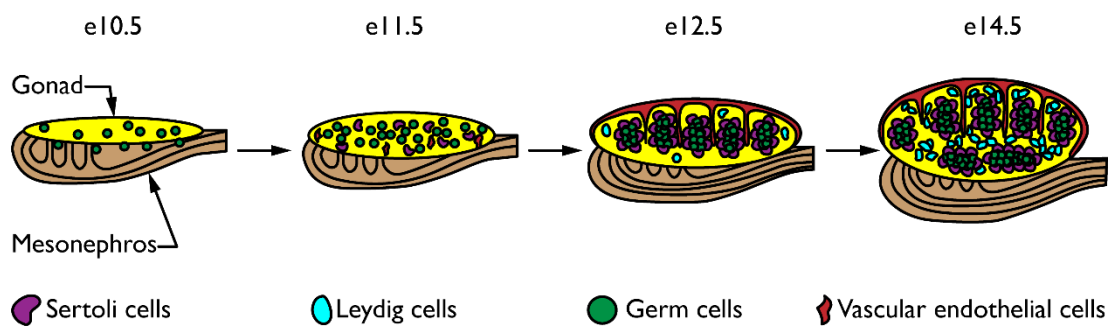


Figure 1.1 Formation of the foetal testis. Germ cells (green) migrate into the developing gonad while the Sertoli cells (purple) differentiate and aggregate around the germ cells. Vascular endothelial cells (red) migrate into the gonad between the cords and the FLCs (blue) differentiate and fill the interstitial compartment. Adapted from (Ross and Capel, 2005).

1.1.2 Sex determination

Sex determination is primarily driven by the presence or absence of the Y chromosome. In the male mouse, *sry* (sex-determining region Y) is expressed in the pre-Sertoli cells of the developing gonad between embryonic day e10.5 and e12 and activates SRY-box 9 (SOX9), which drives the differentiation of this cell type (Burgoyne et al., 1988). A critical threshold of SOX9-expressing Sertoli cells must be reached for male development to proceed as normal (Svingen and Koopman, 2013).

1.1.3 Germ cells

After the germ cells have colonised the gonads they arrest in mitosis. In females retinoic acid induces entry to meiosis, whereas in males retinoic acid is degraded preventing entry into meiosis (Bowles et al., 2006). In rodents primordial germ cells express octamer-binding transcription factor 4 (OCT4) and once they enter the gonad they begin to express mouse VASA homologue (MVH; (Toyooka et al., 2000). The gonocytes are highly proliferative until e13.5 when the rate slowly declines until a period of mitotic quiescence from e16 to just after birth (Vergouwen et al., 1991, Nagano et al., 2000). OCT4 is then down-regulated in all germ cells between e17 and birth (Zayed et al., 2007).

In humans during foetal life there is a heterogeneous population of germ cells with gonocytes and prespermatogonia present together (Anderson et al., 2007). The gonocytes, express pluripotency markers such as OCT4 (Gaskell et al., 2004) and the number of these cells progressively decline as they differentiate into pre-spermatogonia expressing MVH and MAGE-A4 (melanoma-associated antigen 4; (Gaskell et al., 2004, Anderson et al., 2007). The OCT4⁺/MAGE-A4⁻ gonocytes are highly proliferative, while the MAGE-A4⁺/OCT4⁻ prespermatogonia have a lower proliferation rate (Mitchell et al., 2010).

1.1.4 Sertoli cells

The Sertoli cells role in foetal life is to direct masculinisation of the gonad (Sharpe et al., 2003). There are few major changes to the Sertoli cells during this time. They proliferate at a low level and express markers of immaturity such as anti-Müllerian hormone (AMH).

1.1.5 Foetal Leydig cells

The foetal Leydig cells (FLCs) produce testosterone throughout testicular development, which is vital for masculinisation of the foetus (Griswold and Behringer, 2009). FLC origin is not fully understood with potential sources being the mesonephros (Mayerhofer et al., 1996, Nishino et al., 2001) or the coelomic epithelium (Karl and Capel, 1998). Recent evidence points to FLCs being derived from both of these locations (DeFalco et al., 2011). FLC differentiation is driven by signals from the Sertoli cells such as Desert hedgehog (Yao et al., 2002) and platelet-derived growth factor- α (Brennan et al., 2003). In humans the FLCs proliferate and differentiate between 7–14 weeks until they are mature polygonal cells that fill the interstitial space (Svechnikov and Söder, 2008).

1.2 Postnatal testis development

1.2.1 Germ cell development

In rodents the prespermatogonia are located in the centre of the seminiferous tubules and are quiescent at birth. At approximately postnatal day (pnd) 3–5 the germ cells resume proliferation. Potential signals from the Sertoli cells to trigger this process include platelet derived growth factor and 17β -estradiol (Li et al., 1997). Coincident with this, the germ cells migrate to the basement membrane, where they enter the spermatogonial stem cell (SSC) niche (Payne, 2013). Cells that remain in the centre of the tubules and fail to migrate subsequently undergo apoptosis (Payne, 2013). There is a dramatic increase in the number of SSCs within the testis between birth and adulthood (Shinohara et al., 2001). In the rodent the A_{single} SSC acts as both the stem cell and the progenitor cell, whereas in humans the A_{dark} is thought to be the true stem cell and A_{pale} the proliferating progenitor that acts as a functional reserve (Fig. 1.2; (Jahnukainen et al., 2006). This results in different roles for the true stem cells between different species. These SSCs reside within a specific niche created by the Sertoli cells, basement membrane, and vasculature (Yoshida et al., 2007). There are many factors implicated in the regulation of this niche including glial cell-derived neurotrophic factor (GDNF), colony stimulating factor 1 (CSF-1) and fibroblast growth factor 2 (FGF2; (Oatley and Brinster, 2012).

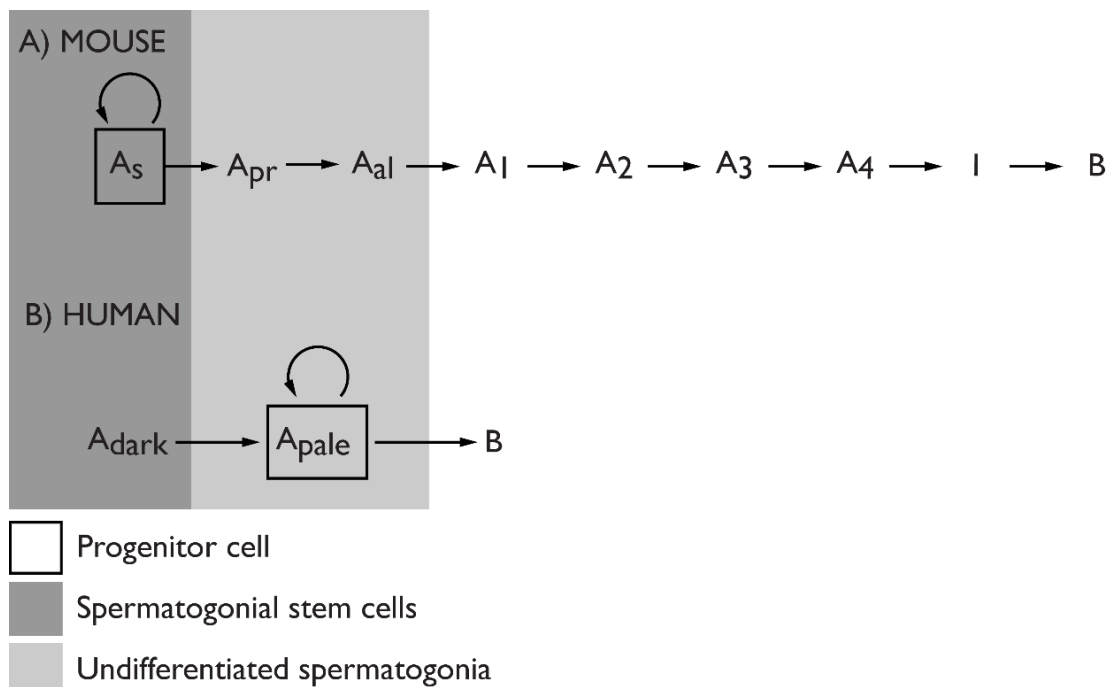


Figure 1.2 SSCs in mouse and humans. A_s = A-single, A_{pr} = A-paired, A_{al} = A-aligned. Adapted from (Jahnukainen et al., 2006).

1.2.2 Sertoli cell maturation

Sertoli cells must proliferate in neonatal life to produce final numbers before terminally differentiating around puberty. This is vitally important, as each Sertoli cell can only support a defined number of germ cells and therefore any decrease in the final number of Sertoli cells will decrease the maximum germ cell producing capacity of the testis (Orth et al., 1988). In humans, this proliferation is thought to occur in two phases, one in the foetal and neonatal period, and the other at the onset of puberty (Murray et al., 2000). This is in contrast to rodents that do not have a 'childhood' period and proliferation continues from foetal life until pnd17 (Vergouwen et al., 1991). The proliferation of Sertoli cells is under the control of androgens and a knockout of the androgen receptor (AR) results in a decrease in the number of Sertoli cells at birth (Johnston et al., 2004). This is despite a lack of the AR in Sertoli cells during this period, and therefore must be an indirect effect through action of other cells in the testis (Sharpe, 2010). The Sertoli cells express different functional markers throughout life, such as AMH in the foetal and neonatal period.

As the Sertoli cells reach terminal differentiation the AR is expressed and AMH is lost (Sharpe et al., 2003). SOX9 is used as a marker for the Sertoli cells in this thesis as it is expressed in both the foetal and neonatal periods and is not affected by developmental stage.

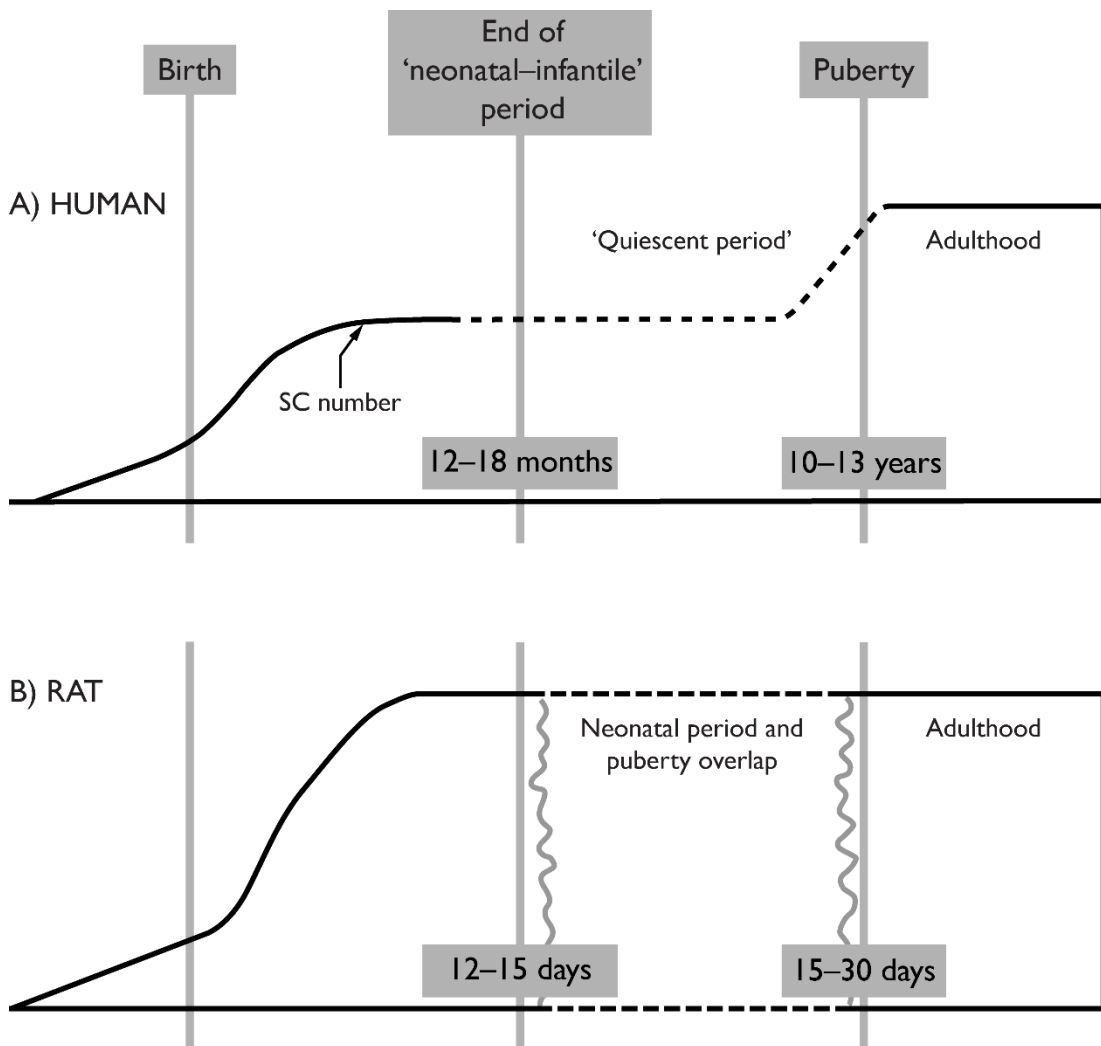


Figure 1.3 Proliferation and maturation of Sertoli cells in the rat and human testis. In the human testis there is an increase in proliferation in neonatal life followed by a quiescent period until puberty. In the rat there is no quiescent period as neonatal and prepubertal periods overlap. Adapted from (Sharpe et al., 2003).

1.2.3 Leydig cell development

The differentiation of adult Leydig cells occurs at puberty from a stem cell population distinct from the FLCs (Fig. 1.4). The adult Leydig cell progenitors proliferate in neonatal life, and then at puberty express steroidogenic enzymes and cease proliferation. In humans the FLCs regress in the first year of life after contributing to the high testosterone levels in neonatal life (Svechnikov and Söder, 2008). Upon stimulation with luteinizing hormone (LH) neonatal Leydig cells proliferate and begin to differentiate into the mature adult Leydig cells (Fig. 1.4; (Habert et al., 2001).

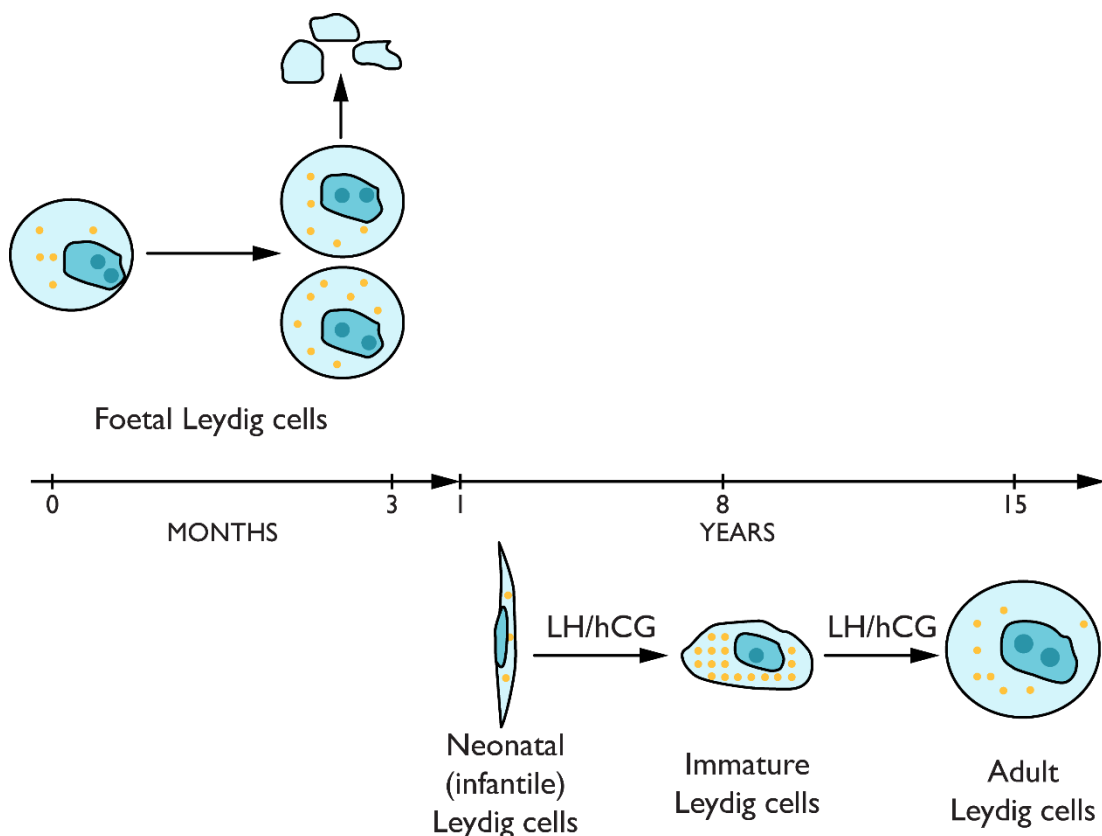


Figure 1.4 Development of the foetal and adult Leydig cells. Early in neonatal life the FLCs degenerate and the neonatal Leydig cells begin to mature. The adult Leydig cells only reach maturity at puberty. Yellow dots indicate cytoplasmic lipid droplets Adapted from (Svechnikov and Söder, 2008).

Chicken ovalbumin upstream promoter transcription factor 2 (COUPTFII) is used extensively in this thesis as a marker of the interstitial cell population. COUPTFII is an orphan nuclear receptor, and ablation during prepuberty results in infertility, as Leydig cell differentiation is arrested (Qin et al., 2008). COUPTFII is present in the majority of interstitial cells in the neonatal testis, but is not found in the tubules. It marks a heterogeneous population of cells. It has been shown that cells that are COUPTFII⁺/3 β -HSD⁻ in the foetal testis are adult Leydig stem cells (ALSCs) and interference with these cells can result in disturbed function of adult Leydig cells in later life (Kilcoyne et al., 2014).

1.3 Spermatogenesis

Spermatogenesis is the process by which spermatogonia differentiate into mature sperm, and begins at puberty. It is composed of three main phases: mitosis, meiosis and spermiogenesis (Fig 1.5). Initially the spermatogonia proliferate and differentiate into primary spermatocytes. These mitotic divisions determine the number of sperm produced, as proliferation stops and each spermatocyte undergoes meiosis to produce four haploid spermatids. Finally, the spermatids undergo the process of spermiogenesis, where they undergo morphological and functional maturation to produce mature spermatozoa. This process is spatially organised in the testis with the more mature stages situated closer to the lumen of the tubule. At this age the Sertoli cells form the blood–testis barrier comprised of tight junctions between adjacent Sertoli cells (Fig. 1.5). This barrier separates the proliferating spermatocytes in the basal compartment from meiosis and spermiogenesis, which occur in the adluminal space (Cheng and Mruk, 2002). Testosterone and follicle stimulating hormone (FSH) are both required for normal levels of spermatogenesis (O'Shaughnessy, 2014), as a double knockout of both the FSH receptors and AR specifically in the Sertoli cells showed a massive reduction in germ cell number and failure to enter meiosis (Abel et al., 2008). This was an additive effect over the single mutants (Abel et al., 2008).

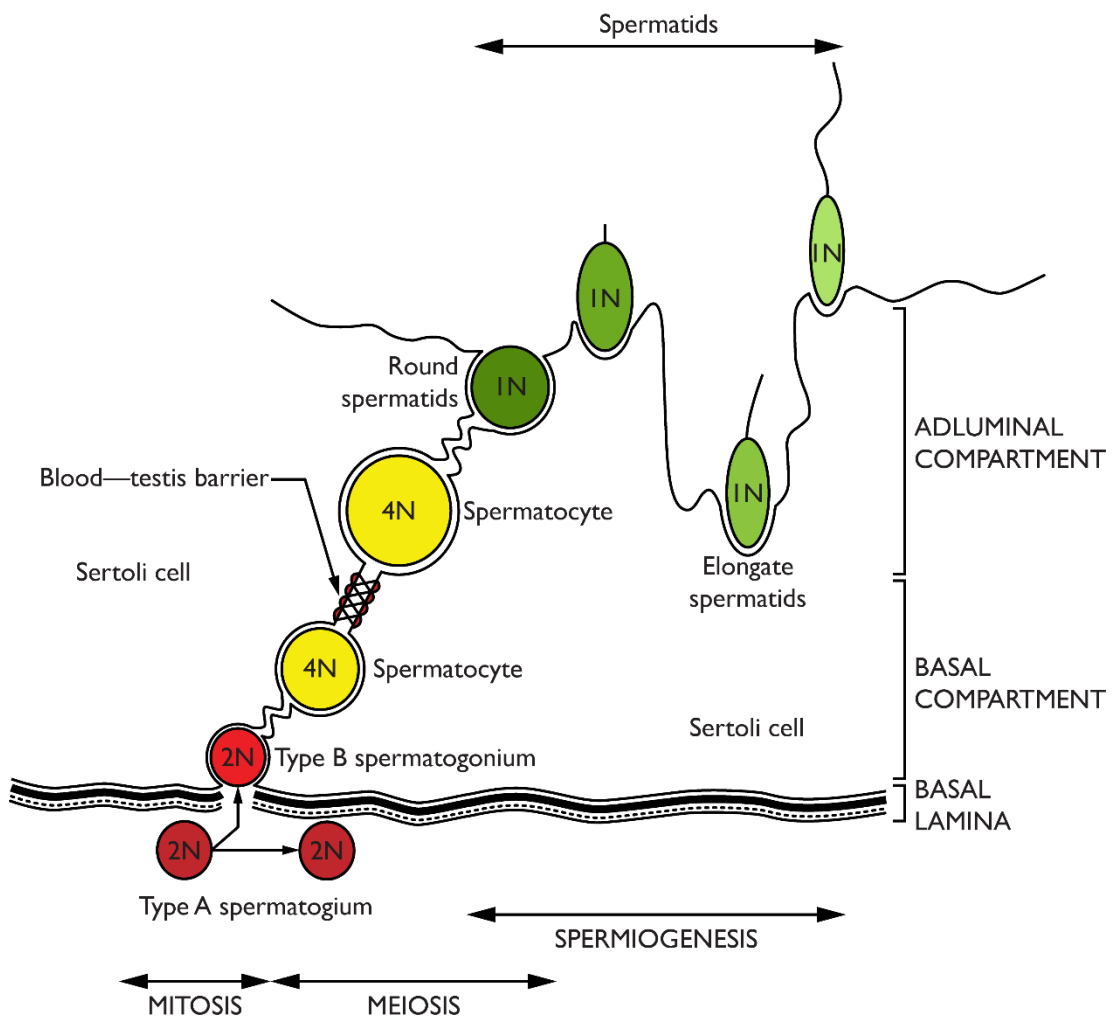


Figure 1.5 Spermatogenesis. The three stages of spermatogenesis are indicated along with the position of the blood—testis barrier separating the basal from the adluminal compartment. Adapted from (Cheng and Mruk, 2002).

1.4 Childhood cancer

In the UK there are approximately 1,600 children aged 0–14 diagnosed with cancer every year. This means 1 in every 500 children will develop a cancer by the age of 14 (Cancer Research UK, 2014). Five-year survival rates have now reached 82% resulting in a large cohort of survivors of childhood cancer (Cancer Research UK, 2014). Nearly one third of all childhood cancers are leukaemia, and the most common solid tumours are brain and spinal tumours (Stiller et al., 2012). Due to the variety of childhood cancers, treatment is also highly variable but is often a combination of surgery, chemotherapy and radiotherapy.

1.4.1 Fertility following chemotherapy

Chemotherapy can cause a variety of adverse side effects in both the long and short term. Infertility can be of concern to both patients and parents of children treated for cancer. Chemotherapy primarily targets dividing cells and therefore the proliferative seminiferous epithelium is particularly vulnerable to chemotherapy-induced damage. The risk of infertility appears related to the specific drugs given (Meistrich et al., 1982) and the cumulative dose received, with alkylating agents thought to be the most gonadotoxic.

The Childhood Cancer Survivor Study has provided long term outcome information from children who survived cancer in the 1970s and 80s, with a large cohort of over 6,000 people (Green et al., 2010). This indicated that children exposed to procarbazine or cyclophosphamide (CYP) were less likely to sire a pregnancy than their siblings (Green et al., 2010). However, this study uses entirely self-reported questionnaire data with no semen or hormone analysis. The St. Jude Lifetime Cohort Study has a much smaller cohort but used semen analysis and indicated that 25% of adult male survivors of childhood cancer had azoospermia with a further 28% presenting with oligospermia (Green et al., 2014). Despite a trend for higher doses of alkylating agent to have an increased chance of impaired spermatogenesis, there was no clear threshold at which this occurred making it difficult to predict clinical outcomes (Green et al., 2014). Only at highest cumulative doses of CYP are men completely sterile (Jahnukainen et al., 2011), with incomplete depletion of the SSCs in boys treated for acute lymphoblastic leukaemia (Nurmio et al., 2009a). If even a few stem cells survive, they can restore fertility long term (Nurmio et al., 2009a). Despite the effect on fertility, psychological assessment of survivors of childhood cancer indicates a similar quality of life between survivors and controls (Zeltzer et al., 2008, Gunn et al., 2013).

The Leydig cells are more resistant to chemotherapy than the seminiferous epithelium (Howell and Shalet, 2001) but, childhood cancer survivors can still suffer from hypogonadism, which can result in depression, disturbed metabolism and cardiovascular disease (Romerius et al., 2009).

1.4.2 Fertility preservation and potential preventative treatments

When adult men undergo gonadotoxic chemotherapy they have the option of cryopreserving a sperm sample prior to treatment, should they wish to have their own genetic children following treatment. This is not available for prepubertal patients, who do not yet produce mature sperm (Valli et al., 2014). A number of different experimental options for preservation are being investigated (Fig. 1.6) including using testis tissue for transplantation (Schlatt et al., 2003, Goossens et al., 2008) or culture to mature sperm *in vitro* (Sato et al., 2011). Or the testis biopsy could be used for SSC enrichment and culture followed by transplantation (Sadri-Ardekani et al., 2011, Hermann et al., 2012). A final potential treatment could be using induced pluripotent stem cells (iPSC) from skin biopsies for differentiation into sperm (Fig 1.6; (Hayashi et al., 2011, Easley et al., 2012). If sperm are produced *in vitro* they would need to undergo intracytoplasmic sperm injection (ICSI) in order to fertilise an oocyte.

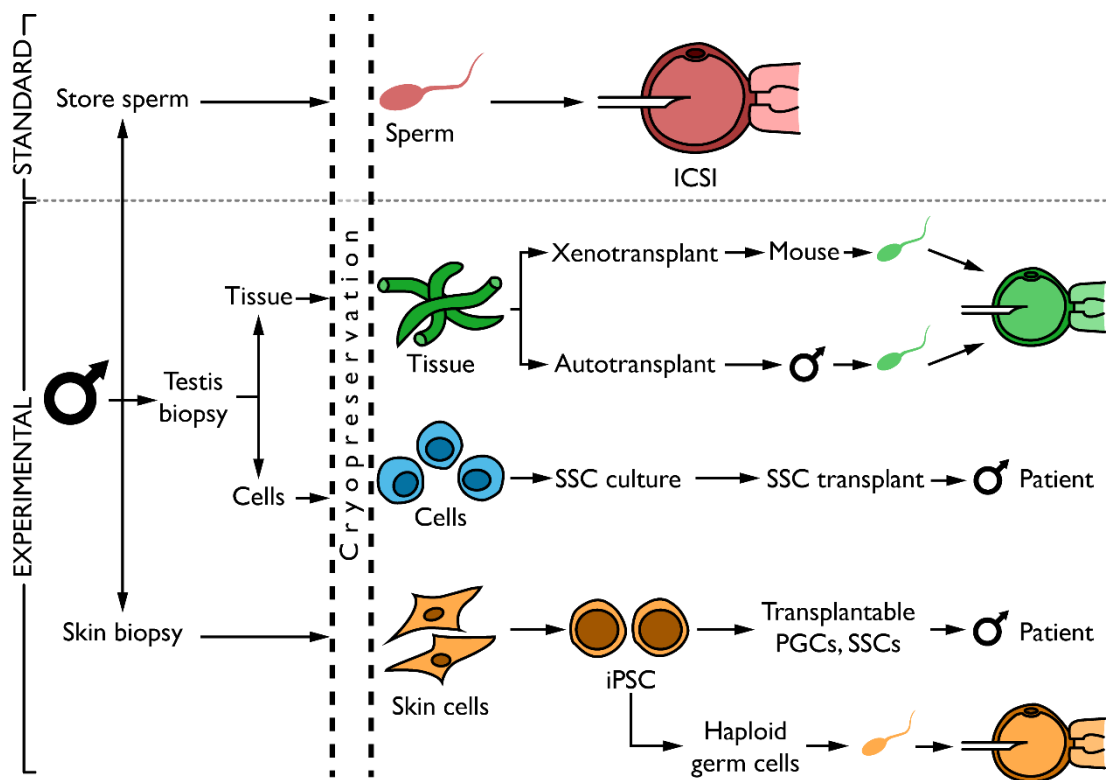


Figure 1.6 Fertility preservation in male patients. The only standard method for fertility preservation is storing sperm followed by assisted reproductive techniques. There are a number of experimental techniques in development. Testis tissue biopsies can provide tissue for transplantation, or culture and cells for culture followed by transplant or in vitro maturation. Skin biopsies could also be taken and iPSCs produced and differentiated into germ cells to be used for transplantation or ICSI. Adapted from (Clark et al., 2011).

Because there are no fertility preservation options for prepubertal boys, efforts have been made to reduce the susceptibility of the testis to chemotherapy induced damage. A lot of research has been done into hormonal methods of preventing the proliferation of the germ cell population and therefore protecting it from cytotoxic agents that target dividing cells. In rats administration of steroid hormones or gonadotrophin releasing hormone protected the SSCs from procarbazine induced death (Parchuri et al., 1993). However, in humans this has proved unsuccessful (Waxman et al., 1987, Thomson et al., 2002, Johnson et al., 1985).

1.5 Chemotherapy

Three chemotherapeutic agents were investigated in this project. CYP was chosen, as it is believed to be the one of the most gonadotoxic and widely used chemotherapeutic agents. Cisplatin (CIS) and doxorubicin (DOX) were also tested, as both are used to treat prepubertal patients and represent two different classes of chemotherapeutics. They have been shown to have different effects on the ovary, with CIS primarily targeting the germ cells, and DOX affecting the supporting granulosa cells (Morgan et al., 2013).

1.5.1 Cyclophosphamide

CYP is an alkylating agent, one of the most successful anti-neoplastic agents developed, and has been used extensively in the treatment of cancer since the late 1950s (Emadi et al., 2009). It is a prodrug that has to be activated in the liver to 4-hydroxycyclophosphamide (Fig.1.7). This then can transform into aldophosphamide which readily dissociates in cells to the active metabolite phosphoramidate mustard (PM), producing acrolein as a by-product (Boddy and Yule, 2000). PM causes DNA crosslinks at guanine residues, which prevent DNA replication (Dong et al., 1995). When this cannot be repaired, cells undergo apoptosis. Aldophosphamide can be inactivated by aldehyde dehydrogenase to carbophosphamide (Fig 1.7) and therefore cells with high levels of aldehyde dehydrogenase are resistant to CYP induced damage (Hilton, 1984).

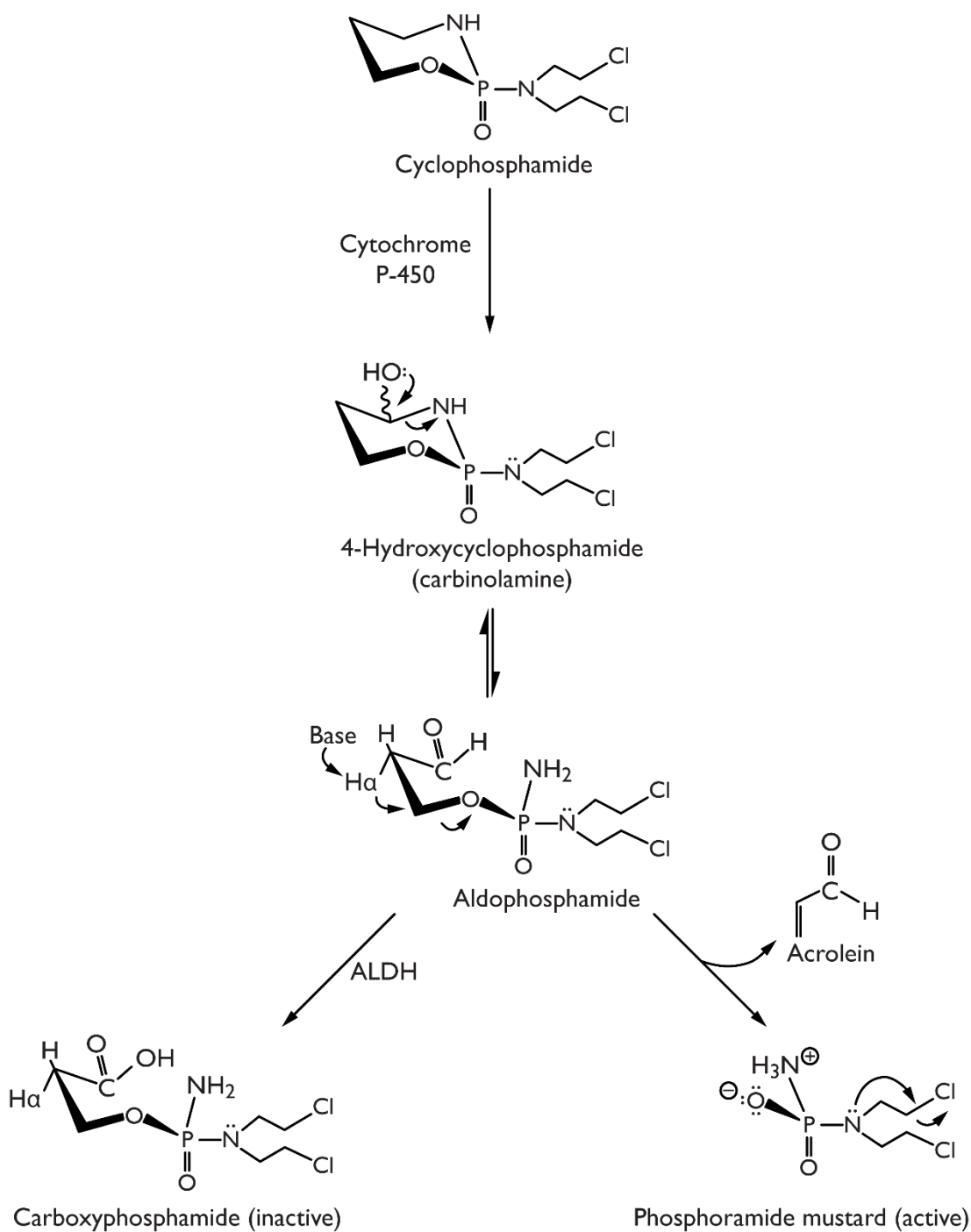


Figure 1.7 Activation of CYP. CYP is activated in the liver to 4-hydroxycyclophosphamide where it transforms into aldophosphamide. This can then be activated into the alkylating compound PM. In cells with a high level of ALDH aldophosphamide is inactivated to carboxyphosphamide. Adapted from (Emadi et al., 2009)

1.5.2 Cisplatin

CIS is a chemotherapeutic drug usually given to combat a variety of cancers including sarcomas, lymphomas and germ cell cancers. It has a platinum core with two amino groups and two chloride ions. When CIS enters a cell, the chloride ions become displaced by water. The resulting product is an electrophile and therefore able to bind to the nucleophiles within the cell, for example as DNA or proteins (Dasari and Tchounwou, 2014). CIS primarily binds to purine bases in DNA and forms intra-strand crosslinks (Wang and Lippard, 2005). These crosslinks produce a physical distortion in the DNA, triggering a whole cascade of downstream cellular effects. This damage will initiate repair mechanisms, such as the nucleoside excision repair, and mismatch repair systems (Gonzalez et al., 2001). However, when the damage is too great for the cell to repair, the apoptotic signalling pathways are activated.

Recently increasing importance has been given to the non-nuclear effects of CIS, as it has been shown that CIS can induce apoptosis when the nucleus is removed from cells (Mandic et al., 2003), and that only 1% of cellular accumulated CIS is found bound to the DNA (Yu et al., 2008). Particular emphasis has focused on oxidative stress targeting the mitochondria of the cell. CIS inhibits antioxidant enzymes and therefore reactive oxygen species can cause mitochondrial dysfunction and release pro-apoptotic factors leading to cell death (Sancho-Martinez et al., 2012). In addition, the endoplasmic reticulum (Mandic et al., 2003) and lysosomes (Sancho-Martinez et al., 2012) have been implicated in the cytotoxicity of CIS. This indicates that there is a whole host of cellular targets for CIS. Once total cellular damage reaches a critical threshold the cell can no longer repair itself and the cell undergoes apoptosis.

1.5.3 Doxorubicin

DOX is an anthracycline antibiotic that has been used in the treatment of cancer for over 50 years and is used in the treatment of solid tumours in childhood (Minotti et al., 2004). DOX has been proposed as having many different mechanisms of action such as inhibition of DNA synthesis (Wang et al., 1972), DNA intercalation (Cullinane et al., 1994), and the generation of free radicals resulting in oxidative stress (Bachur et al., 1977). However, the majority of work has been completed at concentrations greater than 1 μM (Gewirtz, 1999), even though 1–2 μM is the maximum initial concentration observed in patients' serum and falls rapidly to 0.01–0.1 μM (Greene et al., 1983). The most likely mechanism of action is as a topoisomerase inhibitor, as cells that have a reduction in topoisomerase activity are resistant to DOX (Deffie et al., 1989). DOX primarily targets topoisomerase II, which unwinds DNA by transiently forming DNA double strand breaks. DOX stabilises the complex that forms between DNA and topoisomerase II following DNA cleavage, and therefore prevents replication and transcription by leaving double strand breaks unsealed (Tewey et al., 1984). However, it has been noted that there is little DNA damage to account for the degree of cell death, and therefore it has been suggested that the site of double strand breaks may be important and that DOX causes these at specific gene loci, resulting in irreversible damage (Capranico et al., 1990).

1.6 Aims of the thesis

The aims of this project were to:

- To examine the culture of postnatal mouse testis tissue for use in toxicological testing.
- To examine the effect of CYP, CIS or DOX on the prepubertal mouse testis using an *in vitro* culture system.
- To examine the culture of human foetal testis tissue and start investigation into the effect of chemotherapy on this tissue.

Chapter 2. General material and methods

2.1 Animal work

All experiments were approved by the University of Edinburgh ethical review committee and carried out in accordance with UK Home Office regulations. Animals had access to food and water *ad libitum*. C57Bl/J6 or CD1 mice were used for all experiments as indicated in subsequent chapters.

2.1.1 Dissection

Mice were killed by decapitation and the testes dissected into filter sterilised dissection media consisting of Leibowitz L-15 (Invitrogen, UK) solution supplemented with 3 mg ml⁻¹ bovine serum albumin (BSA; Sigma Aldrich Ltd., UK), which had been warmed at 37 °C for at least 30 min prior to use. Whole control uncultured testes were washed in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, UK) before being fixed as described below (see 2.4).

2.1.2 Preparation of mouse testis tissue for culture

Using insulin needles, the testes were cleaned up of any other tissue. Each testis was then dissected using an Altomed blade into 30 to 40 pieces approximately 0.5 mm³ in the dissection media above (see 2.1.1) in preparation for addition to culture medium.

2.2 Media composition

2.2.1 Basic medium

The basic medium was composed of α -minimal essential medium (α -MEM; Invitrogen, UK) supplemented with 10% knockout replacement serum (KRS; Invitrogen, UK). The medium was filter-sterilised and equilibrated at 37 °C and 5% CO₂ for at least 1 h before use.

2.2.2 Enriched medium

An enriched medium was also prepared for some cultures. This consisted of α -MEM supplemented with 1 \times MEM non-essential amino acids (Invitrogen, UK), 2 mM sodium pyruvate (Sigma-Aldrich, UK), 2 mM L-glutamine (Invitrogen, UK), 1 \times ITS (insulin, transferrin and selenium; Sigma-Aldrich, UK), 1 \times Penicillin-streptomycin (Invitrogen, UK) and 10% foetal calf serum (Biosera, UK). The medium was filter-sterilised and equilibrated at 37 °C and 5% CO₂ for at least 1 h before use.

2.3 Chemotherapeutic agents

2.3.1 Cisplatin

Cis-Diammineplatinum(II) dichloride (Cisplatin; Sigma-Aldrich, UK, P4394-25MG) was dissolved in sterile water to prepare an aqueous solution of 1 mg ml⁻¹. This was then serially diluted in sterile water into Eppendorf tubes to produce stock concentrations of 1 mg ml⁻¹, 0.5 mg ml⁻¹ and 0.1 mg ml⁻¹. This was made up prior to culture and stored in the dark for up to 4 weeks. These stock solutions were then added to media to reach the appropriate dilution before addition to the culture.

2.3.2 Doxorubicin

Doxorubicin hydrochloride (Doxorubicin; Sigma-Aldrich, UK, D1515-10MG) was dissolved in sterile water to prepare an aqueous solution of 1 mg ml⁻¹. This was then serially diluted in sterile water into Eppendorf tubes to produce stock concentrations of 0.5 mg ml⁻¹, 0.1 mg ml⁻¹ and 0.05 mg ml⁻¹. This was made up prior to culture and stored in the dark for up to 4 weeks. These stock solutions were then added to media to reach the appropriate dilution before addition to the culture.

2.3.3 Phosphoramidate mustard

PM (Niomech, Germany) was weighed out in a secured Eppendorf tube and media was added to bring the concentration to 0.1 M solution. This was then serially diluted in media to the appropriate concentrations to be added to the culture. PM is

highly volatile and therefore control cultures were performed in separate plates at a different level of the incubator to avoid contamination.

2.4 Tissue fixation and processing

Tissue was washed in DPBS before being placed in 10% neutral buffered formalin solution (NBF; Sigma-Aldrich, UK) for between 90 and 120 min to fix the tissue. Each piece of tissue was placed into microwave-melted 3% agar w/v (Sigma-Aldrich, UK) before being processed and embedded in paraffin wax. The tissue was then sectioned at 5 μm on a microtome and floated out into a water bath heated to 40 °C. Sections were mounted onto coated slides (Thermo Scientific, UK) and dried overnight.

2.5 Haematoxylin and eosin (H&E) staining

Sections were dewaxed in xylene for 2 \times 5 min and rehydrated through decreasing concentrations of alcohols (100%, 95%, 90% and 70%) prior to being washed in water. Slides were then placed in haematoxylin solution for 3 min followed by 15 s in acid alcohol to differentiate and remove background staining. The slides were then placed in Scott's Tap Water Substitute for 2 min to turn the haematoxylin blue to increase the contrast. Finally, the slides were placed in eosin for 3 min before being dehydrated in increasing concentrations of alcohol and cleared in xylene for 5 min. Slides were mounted with DPX (distyrene, a plasticizer, and xylene).

2.6 Immunofluorescence

Slides were dewaxed and rehydrated as previously described for the H&E staining and washed in water.

2.6.1 Antigen retrieval

Antigen retrieval was performed, if required, as noted for individual antibodies (Table 2.1). Slides were placed in 0.01 M citrate buffer (pH 6, Sigma-Aldrich, UK)

and microwaved for 4 × 5 min. Between each set of 5 min the level of citrate buffer was checked and topped up with at most 50 ml of H₂O to ensure that the slides did not dry out. The slides were then cooled for a minimum of 20 min before being washed in phosphate-buffered saline (PBS; Fisher Scientific, UK).

2.6.2 Blocking

Slides were blocked in 20% normal serum diluted in 1 × PBS + 0.1% Tritonx (PBSTx; Sigma-Aldrich, UK) and 5% w/v BSA (Sigma-Aldrich, UK) for 1 h at room temperature. The appropriate serum was used depending on the species in which the secondary antibody was raised.

2.6.3 Primary antibodies

The primary antibodies were appropriately diluted in blocking serum and incubated with the slides overnight at 4 °C in a humidified box. For double immunofluorescence both the primary antibodies were incubated together. One section was incubated with blocking serum alone to act as a negative control.

Table 2.1 Primary antibodies and conditions used.

Antibody	Company	Species	Dilution	Retrieval
MVH	Abcam	Mouse	1:100	Yes
CC3	Cell Signalling Technology	Rabbit	1:500	Yes
SOX9	EMD Millipore	Rabbit	1:100	No
BrdU	Abcam	Rat	1:500	Yes
COUPTFII	R&D Systems	Mouse	1:200	Yes
OCT4	Abcam	Goat	1:200	Yes

CC3: Cleaved Caspase-3; BrdU: 5-bromo-2'-deoxyuridine;

2.6.4 Secondary Antibodies

Slides were washed for 3 × 5 min in PBSTx before being incubated with an appropriate secondary antibody diluted 1:200 in blocking solution. Secondary antibodies were either a biotinylated secondary (Vector Laboratories, USA), to allow amplification before visualisation, or a direct fluorescent Alexa Fluor IgG₁ (Invitrogen, UK). Further details of this process are given in individual chapters. For

double immunofluorescence one of each type of secondary was used. For this and all subsequent steps slides were kept in the dark.

2.6.5 Visualisation

Following incubation with secondary antibody slides were again washed in PBSTx for 3×5 min. The visualisation reagent (streptavidin conjugated fluorescent labels; Invitrogen, UK) was added at 1:200 diluted in blocking solution for 30 min. This step was omitted for single immunofluorescence when a direct Alexa Fluor conjugated secondary antibody was used.

2.6.6 Counterstaining and mounting

Slides were washed for a further 2×5 min in PBSTx before being incubated with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, UK) diluted at 1:5000 for 10 min. Sections were then quickly washed in PBS before being mounted with hard-set VECTASHIELD (Vector Laboratories, USA) and kept in the dark at 4 °C until image acquisition.

2.7 Image acquisition

H&E images were taken with a DMLB Leica microscope integrated with a Leica DFC480 camera (Leica, UK). Fluorescent images were taken on Leica DM5500B microscope and captured with a DFC360X camera (Leica, UK).

2.8 Image analysis

All image analysis was performed using ImageJ. Section areas and tubule areas were calculated by manually selecting the section, or surrounding all of the tubules within an image with the selection tool, and measuring the area in pixels. For tubule diameters a line from one basement membrane to the opposing membrane was drawn and its length was measured. This was only done for complete tubules

within the image. When the tubules were not round, the shortest diameter was measured (Fig. 2.1).

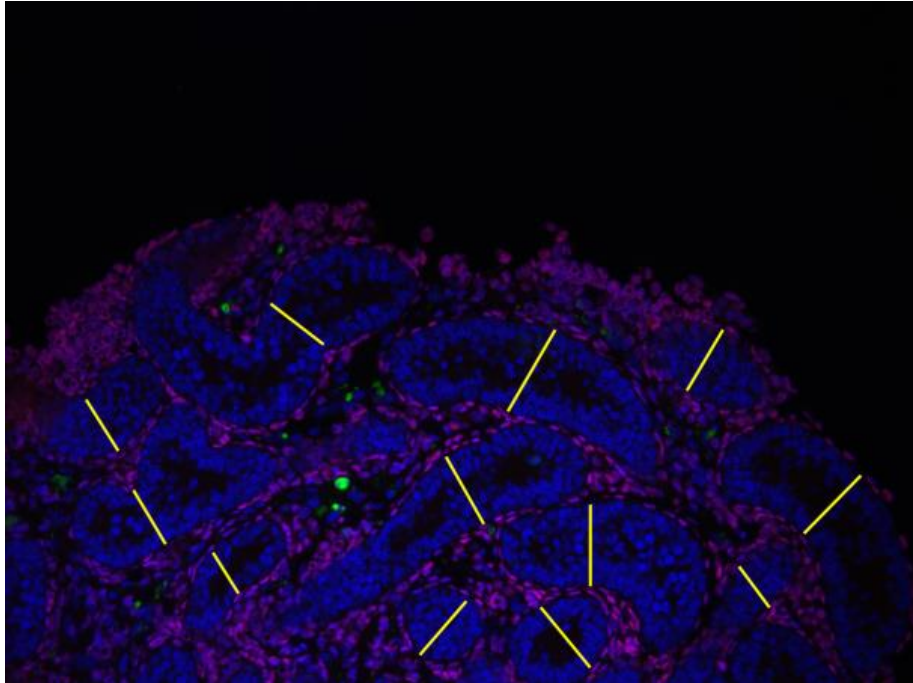


Figure 2.1 Calculation of tubule diameter. Tubule diameter (yellow lines) was calculated by drawing a line across the seminiferous tubules and measuring its length.

In order to calculate the area of the immunofluorescent staining, the captured image was split into individual channels (greyscale representations of fluorophore colours making up the image; Fig. 2.2A). A threshold was then be selected, effectively filtering out the background of the image and leaving only the stained areas of interest (Fig. 2.2B). This threshold filter would then be used to convert the photograph into a binary image (Fig. 2.2C), with the background being ignored (binary 0 value) and the foreground staining the only present pixels (binary 1 value). The area of staining could then be measured by selecting the converted image and using the inbuilt ImageJ pixel counting tool. A single threshold value was used for the analysis of all pieces within a single run of the immunofluorescent staining. If the selected threshold (Fig. 2.2C) resulted in a significant amount of image background not being filtered or if a lot of the staining of interest was

filtered, the threshold was adjusted to a more appropriate value for that specific image. Each cultured testis fragment or whole fixed testis was counted as $n = 1$.

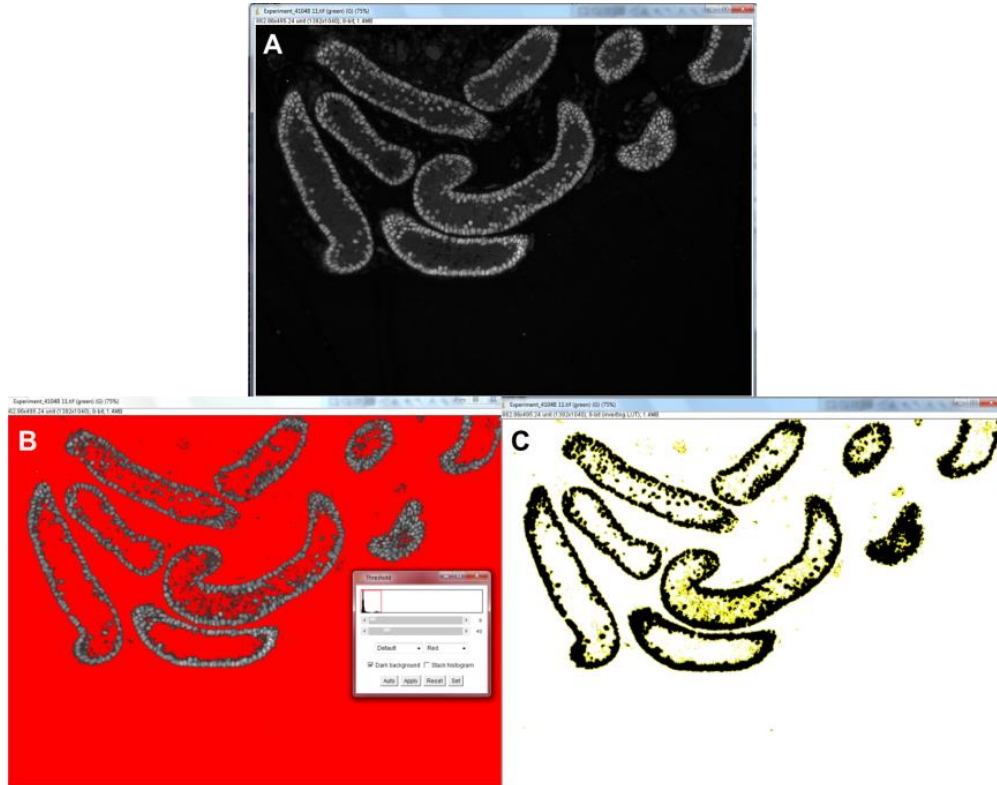


Figure 2.2 Analysis of fluorescent images. A) Single fluorophore image after channels had been split. B) A threshold is selected to separate staining (grey) from the image background (red). Finally C) the image is converted into a binary image where the background is discarded (white) and staining remains. This allows the selection of only the staining area and the calculation of area.

Chapter 3. Culture of postnatal mouse testis tissue

3.1 Introduction

Testicular organ culture is an *in vitro* technique that has been used in many different experimental settings. In comparison to cell cultures organ culture maintains the tissue architecture and the cell signalling required for normal testicular development. Organotypic testicular cultures date back to the 1920s and have been developed for foetal, postnatal and adult tissue in a variety of species.

3.1.1 Membrane culture

Culturing on a membrane has been the chosen method for culturing the neonatal mouse ovary in the lab. It sustains primordial follicle initiation and growth and has been utilised to examine the effects of chemotherapeutic agents (Morgan et al., 2013, Lopes et al., 2014). This technique has also been used to culture rat foetal and neonatal testis tissue (Habert et al., 1991, Hallmark et al., 2007, Li and Kim, 2003). It is thought to benefit the tissue as it is maintained at the interface between the medium and air allowing access to oxygen and medium.

3.1.2 Culture on an agar block

Culture on an agar block has been used extensively in embryonic gonad studies, mainly used for studying sex determination and testis formation (Martineau et al., 1997, Combes et al., 2009). Culturing on agar block has also been established to investigate both hormonal and non-hormonal signalling in neonatal testicular development (Meehan et al., 2000, Zhou and Hutson, 1993). This method is believed to provide more structural support to the tissue and therefore is good for more delicate tissue.

Using agar blocks for testicular culture was initially proposed for *in vitro* spermatogenesis in the 1950s, but was abandoned as there was no initiation of meiosis (Steinberger and Steinberger, 1970), and there has been increasing focus

placed on cell co-cultures rather than tissue culture. Recently, this technique has been further developed (Gohbara et al., 2010) and refined (Sato et al., 2011) to generate elongated haploid spermatids, which, when cultured for up to 60 days, could undergo ICSI and produce live offspring.

3.1.3 Culture in hanging drops

Hanging drops were originally developed for the culture of embryonic stem cells (ESCs). These form embryoid bodies via the aggregation of ESCs at the round bottom of the drop (Kurosawa, 2007). These embryoid bodies differentiate into committed cell types and have been used extensively as a tool to study the development of different tissues (Desbaillets et al., 2000).

Culturing in hanging drops is a potential method for culturing testis tissue as it uses few resources and maintains the architecture of the tissue. This has been developed for mouse embryonic gonads and isolated adult seminiferous tubules (Szczepny et al., 2009), along with adult and foetal human testis tissue (Jorgensen et al., 2014, Jorgensen et al., 2015). In both of these reports, exogenous compounds were added to the cultures in order to manipulate specific signalling pathways. Therefore, hanging drop cultures could be a suitable model for testing the effect of exogenous agents on the testis.

3.2 Aims

The aims of this chapter were:

- To compare the different methods of culturing the postnatal mouse testis in order to evaluate the appropriate methodology for subsequent work.
- To compare the cultured testis tissue to testis development *in vivo*.

3.3 Methods

3.3.1 Animals

Initial cultures on membranes or agar blocks were performed with CD1 mice, whereas hanging drops were performed separately with C57Bl/J6 mice. C57Bl/J6 mice were used for the *in vivo* comparison experiments.

3.3.2 Postnatal testis culture

Following dissection (see 2.1.1 and 2.1.2) testes pieces were placed either basic media (see 2.2.1) or enriched media (see 2.2.2) and cultured in the following methods.

3.3.2.1 Membrane

A Millipore membrane (Camlab Ltd., UK) was placed on 1 ml of medium in a 24 well plate (Greiner Bio-one, UK). A single piece of tissue was placed on each membrane using a sterilised, BSA coated glass pipette. After 48 h (Day 2) in culture 0.5 ml of medium was removed from each well and replaced with fresh medium.

3.3.2.2 Agar

Small (10–20 mm) blocks of 2% agar (Sigma Aldrich Ltd., UK) diluted in PBS were prepared, sterilised and placed into 35 mm culture dishes. Medium was added to fill dish to the top of the block and left to equilibrate for 30 min to replace the PBS. Medium was then changed before the addition of three testis fragments to each agar block. Each day the level of medium was checked and topped up to the top of the agar block if required.

3.3.2.3 Hanging drop

25 µl of medium was placed on the lid of either a six well plate or a Petri dish with approximately 1 ml of DPBS placed in the bottom of the wells or dish to prevent evaporation. Each testis fragment was placed into the drop using a glass pipette and

then the lid inverted to allow the tissue to sink to the bottom of the drop. Testis fragments were transferred to a new plate with new drops of fresh medium after 48 hours.

After four days of culture using the methods outlined above, the tissue was fixed and processed (see 2.4).

3.3.3 Haematoxylin & eosin staining

H&E staining was performed as described (2.5).

3.3.4 Immunofluorescence

Immunofluorescence was performed as described (2.6) with antibody combinations as detailed in Table 3.1.

Table 3.1 Antibodies and combinations for immunohistochemistry

Primary 1	Dilution	Secondary 1	Detection	Primary 2	Dilution	Secondary 2	Retrieval
CC3	1:500	G α -R Bio	Strep 488nm	MVH	1:100	G α -M 568nm	Yes
MVH	1:100	G α -M 568nm	N/A	N/A	N/A	N/A	Yes
SOX9	1:500	G α -R Bio	Strep 488nm	N/A	N/A	N/A	No
COUPTFII	1:200	G α -M Bio	Strep 568nm	CC3	1:500	G α -R 488nm	Yes

G α -R Bio = Goat anti rabbit Biotinylated

G α -M Bio = Goat anti mouse Biotinylated

G α -M 568nm = Goat anti mouse IgG₁ 568nm

G α -R 488nm = Goat anti rabbit IgG₁ 488nm

Strep = Streptavidin

3.3.5 Image acquisition and analysis

Images were acquired and analysed as described (2.7 and 2.8).

3.3.6 Statistical analysis

Statistical analysis was performed with GraphPad Prism. Analysis was based on at least five testis fragments or whole testes. The data were analysed with an analysis of variance (ANOVA) if the data followed a normal distribution, followed by Tukey's *post hoc* test if the ANOVA was statistically significant to compare each group with each other. Where the data were not normally distributed a Kruskal-Wallis test was performed followed by Dunn's multiple comparison tests. All results are given as mean \pm SEM with statistical significance set at $p < 0.05$.

3.4 Results

3.4.1 Neonatal mouse testis culture: membrane, agar, hanging drop

3.4.1.1 General morphological analysis

A general assessment of the culture techniques was made from H&E staining. The tubules were maintained when cultured by any of the methods with a clear flattened layer of PTMs surrounding the tubules (Fig. 3.1). The tissue cultured in hanging drops developed a spheroid shape, whereas cultures on a membrane or agar block flattened out. Some pyknotic cells were found in most of the sections examined; these were mainly toward the centre of the sections (Fig. 3.1).

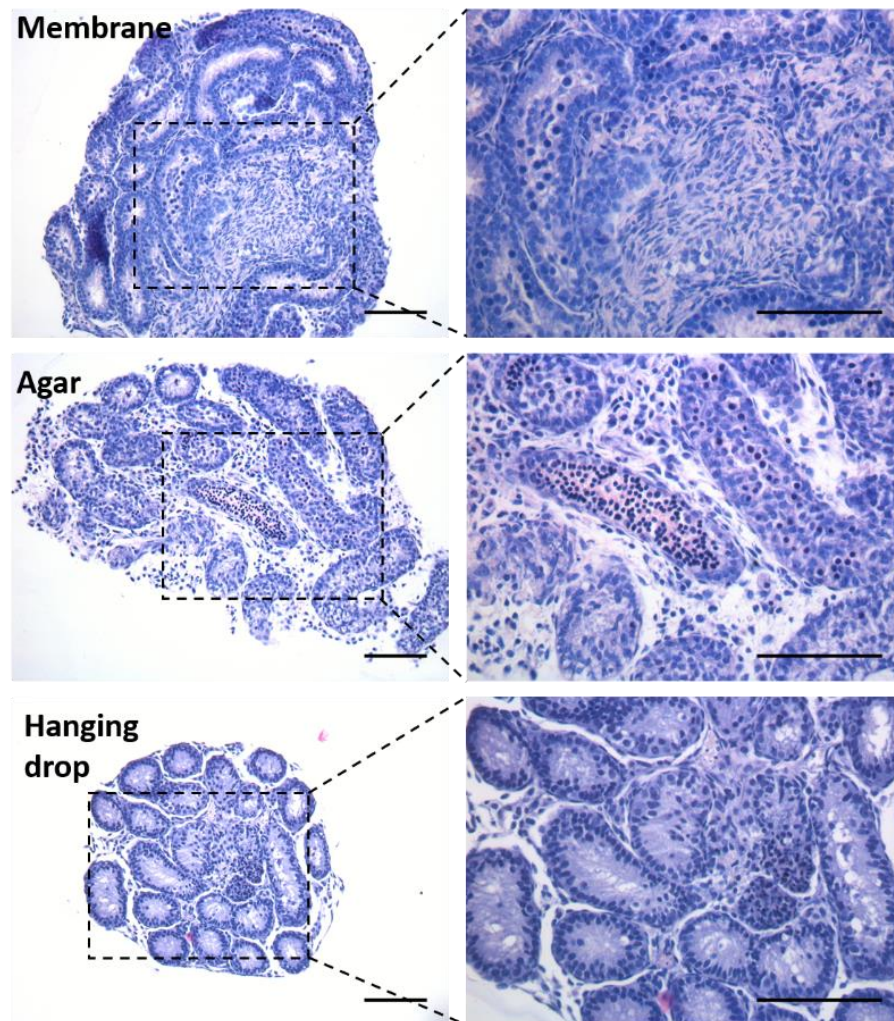


Figure 3.1 H&E culture of postnatal mouse testicular tissue by three methods. The general structure of cultures on membranes, agar or in hanging drops were compared. Scale bars = 100 μm .

The area that the tubules occupied as a proportion of the section area tended to decrease in the hanging drop cultures (Fig. 3.2a) but this did not reach statistical significance ($p=0.078$). The average tubule diameter was not affected by any of the culture techniques (Fig. 3.2b).

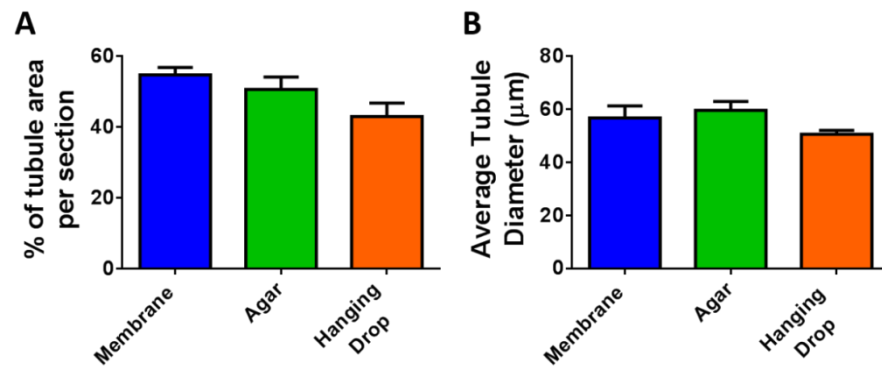


Figure 3.2 Quantification of tubule properties in the cultured tissue. No significant differences were seen between culture techniques for A) the proportion of section that was tubules or B) the average tubule diameter. Bars represent mean \pm SEM with $n = 5$ for membrane and hanging drop cultures and $n = 6$ for agar culture. $p > 0.05$.

3.4.1.2 Germ cells and apoptosis in cultured testicular tissue

In order to investigate whether the culture systems support germ cell development and the health of the tissue, sections were assessed with double immunofluorescence for MVH and CC3. MVH is expressed in all germ cells in the postnatal testis. CC3 is an effector caspase and part of the apoptotic pathway. Germ cells were supported with all culture techniques and there was little apoptosis in both the agar and membrane cultures. The hanging drops, however, showed high CC3 expression in the centre of each section (Fig. 3.3).

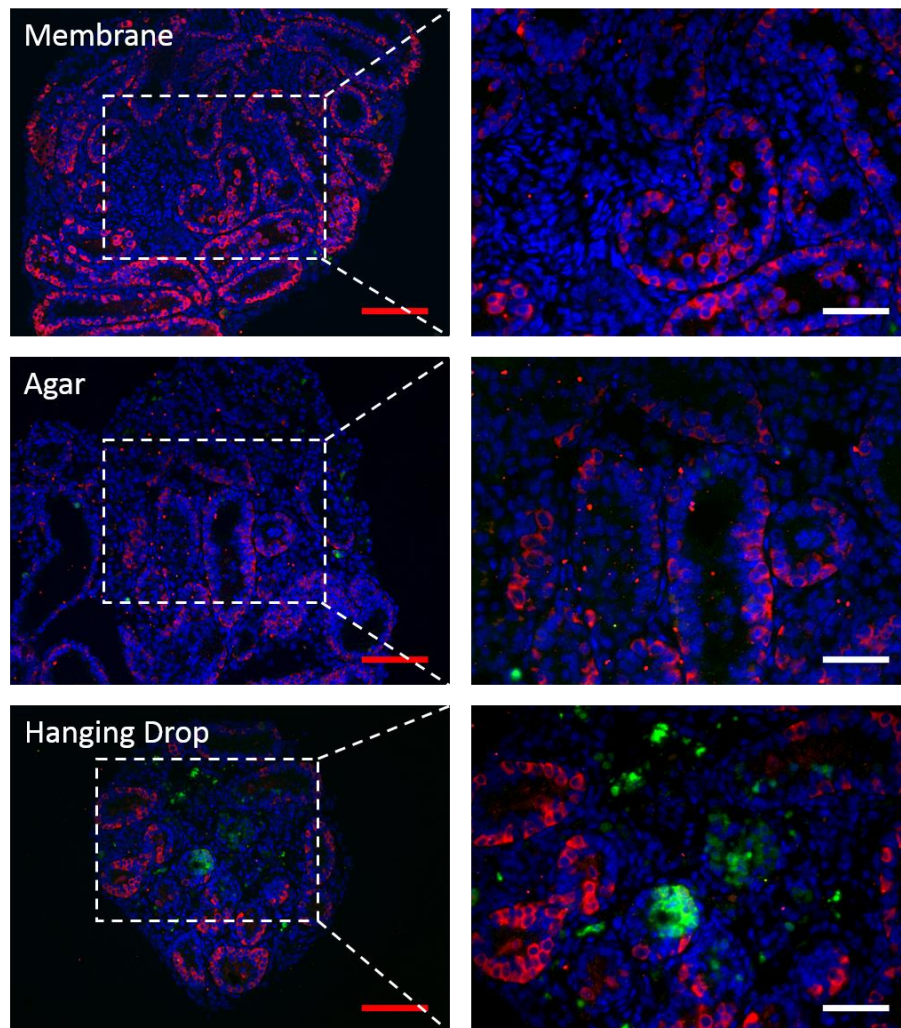


Figure 3.3 Expression of MVH and CC3 in postnatal mouse testis cultured on a membrane, on agar or in a hanging drop. MVH (red) marked germ cells within the seminiferous tubules. There was little CC3 (green) except in the hanging drops. Sections were counterstained with DAPI (blue). Scale bars, red = 100 μm , white = 50 μm .

The level of MVH was quantified and expressed relative to the area of the tubules in the section. This showed a trend towards a decrease in MVH expression in both the agar and hanging drop cultures relative to the membrane culture, but this was not significant ($p = 0.17$, Fig. 3.4).

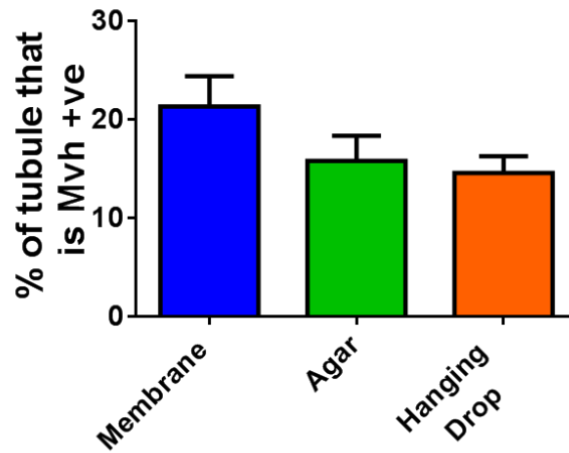


Figure 3.4 Quantification of germ cells in the mouse postnatal culture. Bars represent mean \pm SEM with $n = 5$ for each technique.

CC3 was quantified relative to the area of the section, as it was present in both the tubules and interstitium. There was little CC3 expression in the membrane and agar cultures but the hanging drops showed a much higher level of CC3 (Fig. 3.5).

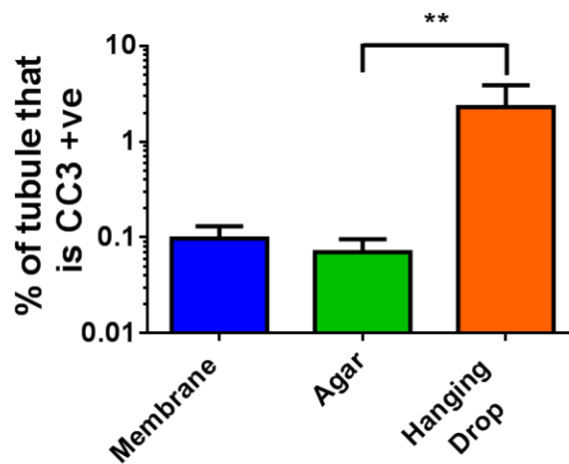


Figure 3.5 Quantification of apoptosis in the mouse postnatal culture. Data was not normally distributed and was analysed with Kruskal-Wallis test followed by Dunn's multiple comparison test. Bars represent mean \pm SEM with $n = 5$ for each technique, ** $p < 0.01$.

3.4.2 Media comparison for hanging drop cultures

Despite the hanging drops showing a high level of CC3 centrally this method has been examined in more detail. This is because it has been used previously to culture human foetal testis tissue. Therefore pnd5 testis fragments were cultured in hanging drops using either the basic medium used for all other techniques or an enriched medium developed for culturing human foetal testis. The enriched media showed no improvement over the basic media with both showing central cell death (Fig. 3.6). In many cases the new media appeared worse with a larger area of pyknotic cells.

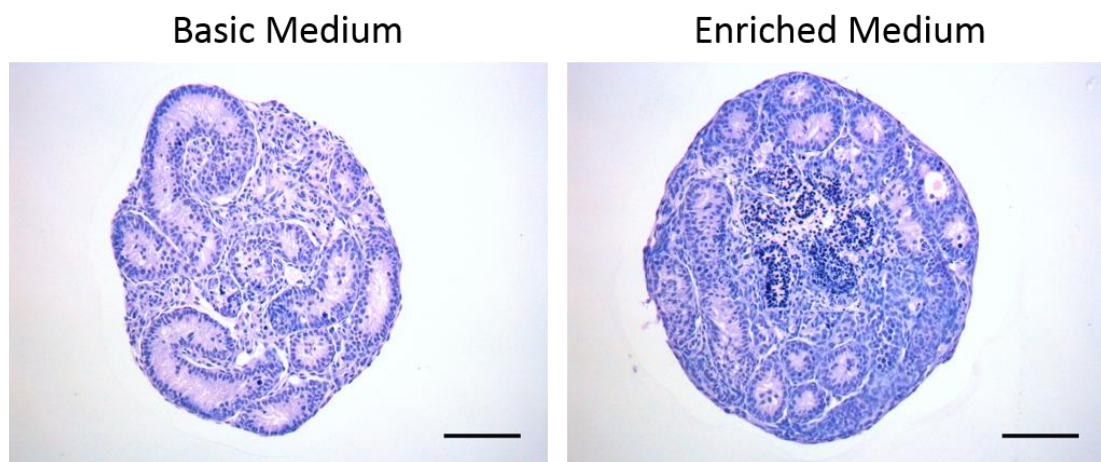


Figure 3.6 Comparison of media for mouse postnatal hanging drop cultures. H&E stain of hanging drops cultured with a basic or enriched medium. Scale bars = 100 μ m.

3.4.3 Summary of method comparison

When all results from the previous sections (see 3.4.1 and 3.4.2) were put together, this indicated that the hanging drops were not suitable for the culture of mouse postnatal testis tissue. There was no significant differences between the agar and membrane cultures. The agar culture is more labour and resource intensive and therefore all subsequent mouse cultures were performed on a membrane.

3.4.4 Cultured testicular tissue in comparison to *in vivo* time points

3.4.4.1 General morphological analysis

Testis fragments cultured for four days on a membrane were compared to two *in vivo* time points to assess whether the culture system supported testicular development. Pnd5 testis tissue was used as a pre-culture control and this was compared with pnd8 which assumes that culturing produces an approximately one day delay in development. There were few morphological differences observable from pnd5 to pnd8 in the mouse testis. The germ cells at pnd5 were very obvious, due to larger size and large volume of cytoplasm compared to the Sertoli cells. There was less difference between the two cell types by pnd8 (Fig. 3.7). It was hard to identify germ cells in the cultured tissue. The tubule diameter and the proportion of the section consisting of tubules increased from pnd5 to pnd8, but this was not observed in the cultured testis tissue (Fig. 3.8).

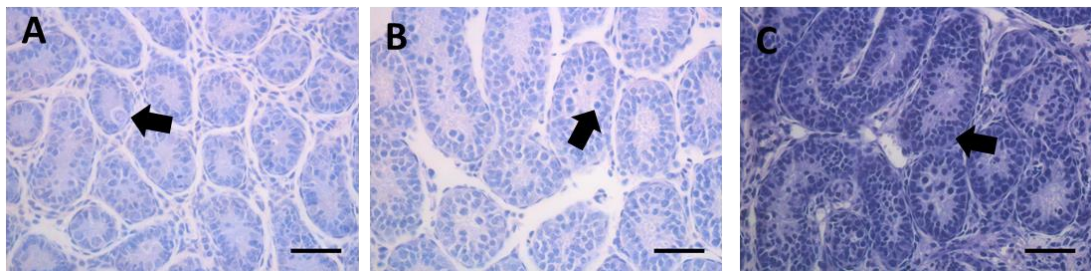


Figure 3.7 H&E of postnatal testis tissue in comparison to cultured tissue. A) pnd5 B) pnd8 and C) cultured testis fragment. Arrows indicate germ cells. Scale bars = 50 μm .

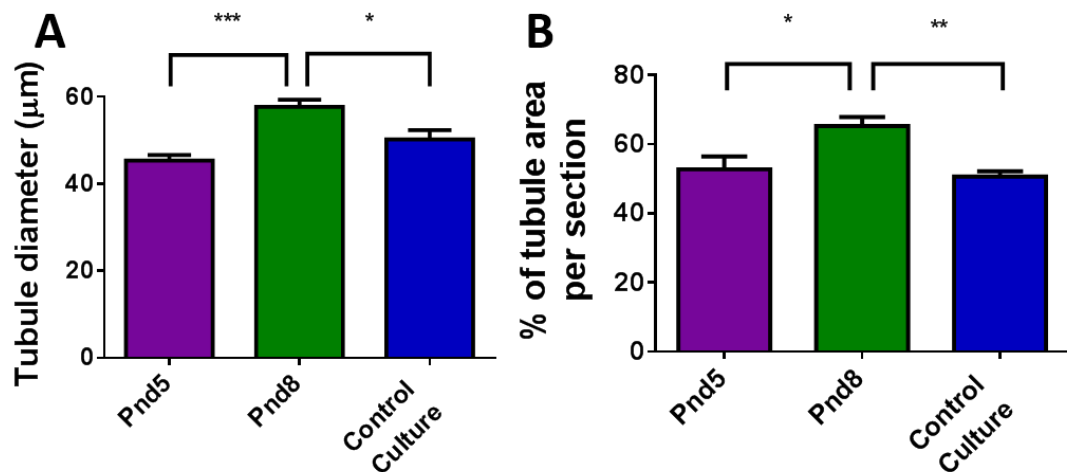


Figure 3.8 Quantification of tubule properties of postnatal mouse in comparison to cultured tissue. There was a statistically significant difference between A) the proportion of tubules per section and B) average tubule diameter. Bars represent mean \pm SEM with $n = 5$ for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4.4.2 Seminiferous tubule composition

The proportion of the tubule containing MVH-positive germ cells increased between pnd5 and pnd8 and there was a trend to this increase in the cultured tissue, although this did not reach significance (Fig. 3.9). There was little difference in the amount of SOX9 expression between the pnd5 and pnd8 testes. The control cultures had a reduced percentage of the tubules that were SOX9-positive (Fig. 3.10). COUPTFII marks the majority of the interstitial cells in the testis including the ALSCs. The proportion of the interstitium containing COUPTFII-positive cells did not change from pnd5 to pnd8 and this was maintained in the control cultured tissue (Fig. 3.11).

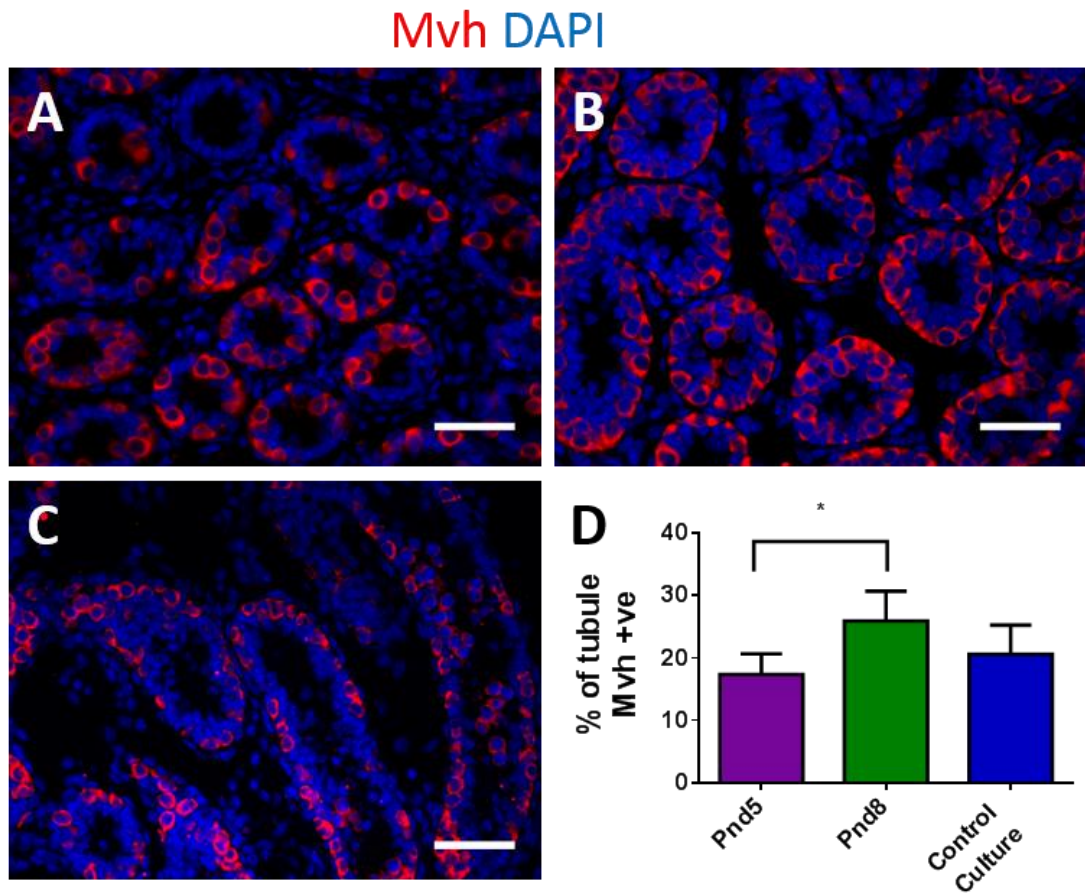


Figure 3.9 Expression of MVH in the postnatal mouse testis. A) pnd5, B) pnd8 and C) control cultured testis. D) Quantification of MVH expression. Bars represent mean \pm SEM with $n = 5$ for each group. $*p < 0.05$

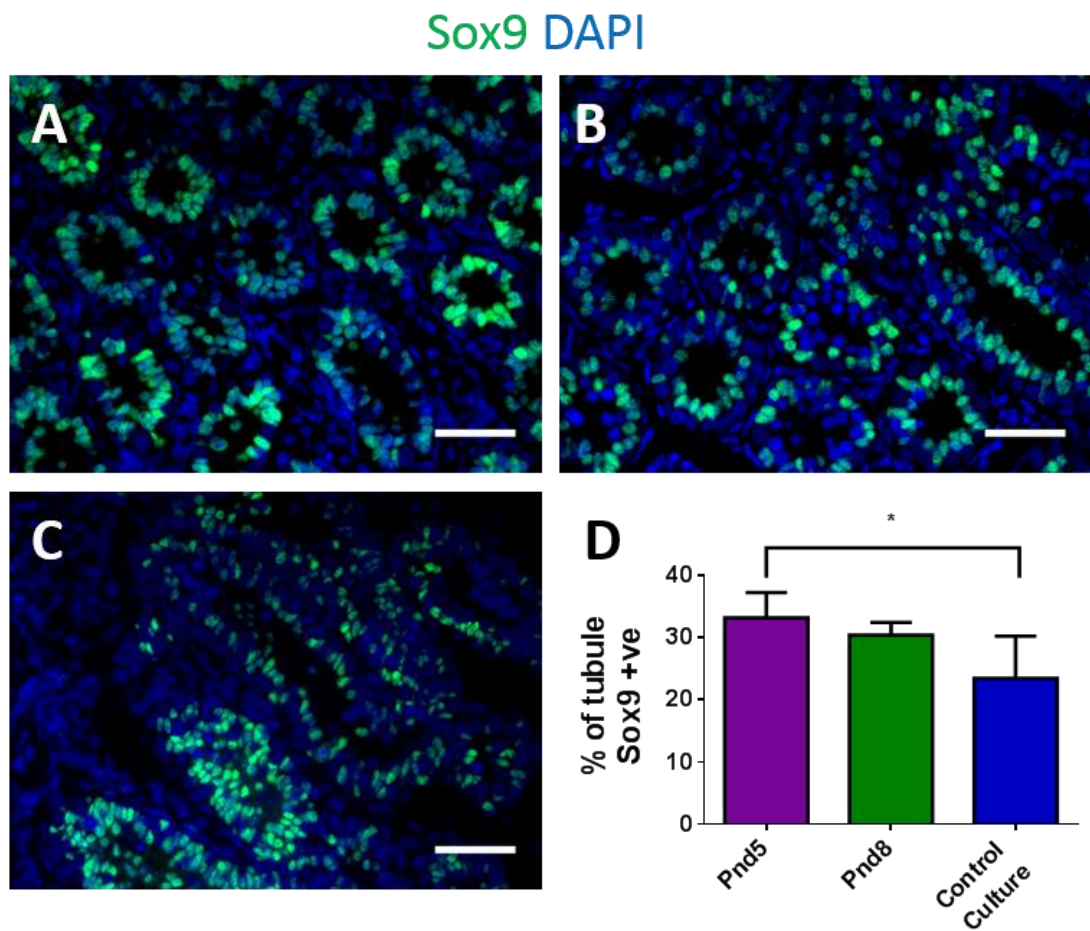


Figure 3.10 Expression of SOX9 in the postnatal mouse testis. A) pnd5, B) pnd8 and C) control cultured testis. Scale bars = 50 μ m. D) Quantification of SOX9 expression. Bars represent mean \pm SEM with n = 5 for each group. *p < 0.05

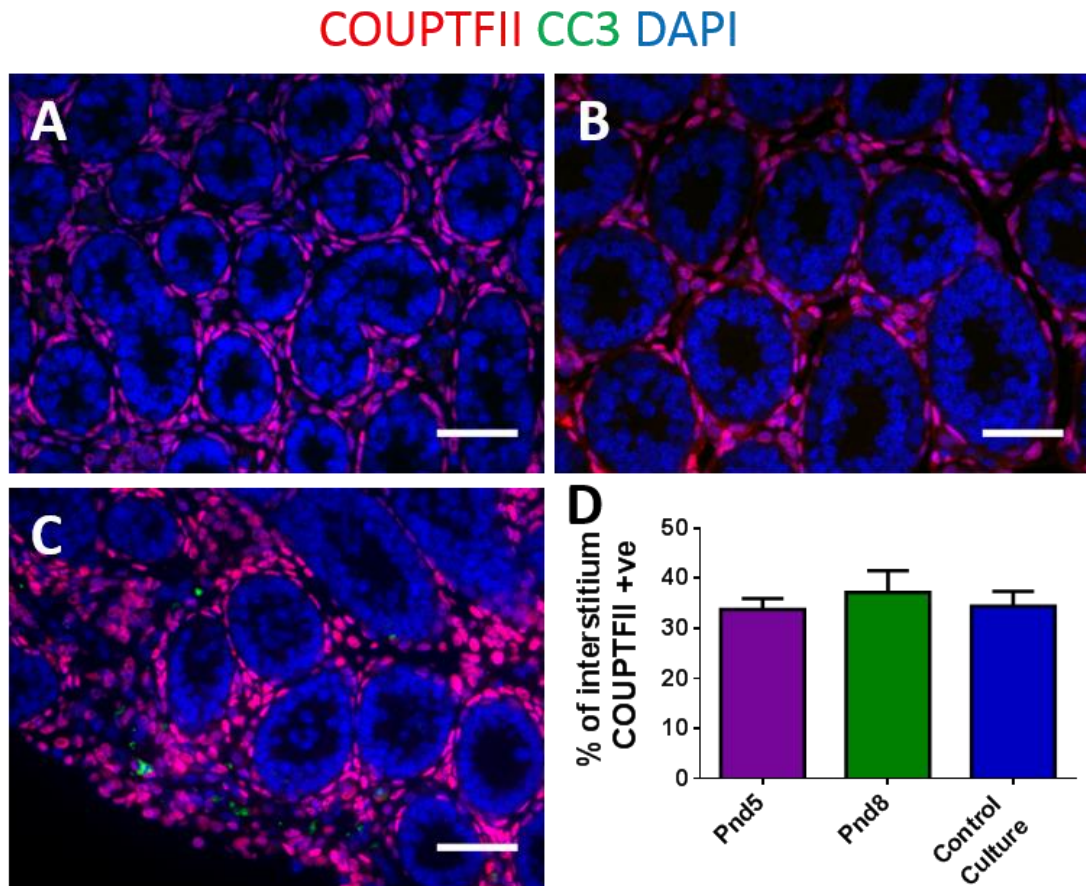


Figure 3.11 Expression of COUPTFII and CC3 in the postnatal mouse testis. A) pnd5, B) pnd8 and C) control cultured testis. Scale bars = 50 μ m. D) Quantification of COUPTFII expression. Bars represent mean \pm SEM with n = 5 for each group.

The cultured tissue had significantly more CC3 expression than the fixed testis tissue (Fig. 3.12). However, this was still a very small percentage of the total area and therefore was deemed an acceptable level of apoptosis.

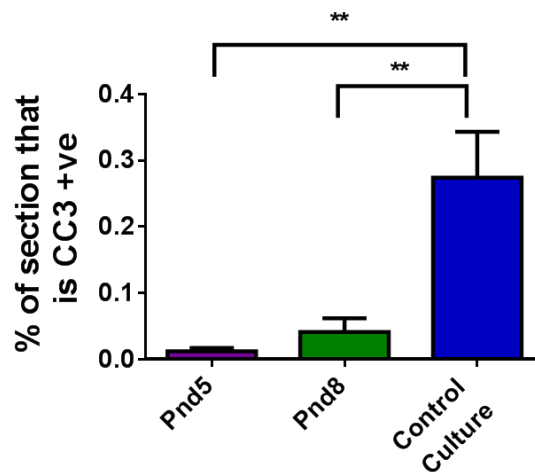


Figure 3.12 Quantification of CC3 expression in the postnatal mouse testis. There was an increase in CC3 expression in cultured testis $p < 0.01$. Bars represent mean \pm SEM with $n = 5$ for each group. $**p < 0.01$

3.5 Discussion

There appeared to be little difference between culturing on membrane and on agar blocks. The hanging drop cultures showed central apoptosis and this was not improved by using an enriched media. This was surprising as this protocol has been used successfully to culture human foetal tissue (Jorgensen et al., 2015). It would be interesting to test if this difference was due to species (mouse *v* human) or age of the tissue (embryonic *v* postnatal). To accomplish this embryonic mouse tissue of an equivalent age to human tissue could be cultured in hanging drops to see if this technique is better at supporting embryonic tissue.

Successful murine hanging drops have only been reported over 48 hour culture, suggesting the four day culture is too long for this technique (Szczepny et al., 2009). These cultures were also using very small testes (e11.5) or isolated seminiferous tubules (Szczepny et al., 2009). The fragments presented here may have been too large to promote survival. Subsequent experiments using mouse postnatal testis tissue were cultured on membranes in the basic media due to lower labour and media requirements compared to the agar culture.

The culture on membrane was shown to support many of the characteristics of the neonatal mouse testis. As there are no major changes to the cell populations and structure of the testis over the time period examined from pnd5 to pnd8/9, the main function of the culture is to maintain the different cell types and structure. There was an increase in the ratio of total of tubule area to germ cell area, along with an increase in the diameter of the testis from pnd5 to pnd8. After birth the germ cells migrate from the centre of the tubules to the seminiferous epithelium where they resume proliferation (Jarvis et al., 2005) and therefore an increase in germ cell proportion during culture would be expected. The Sertoli cells should also be proliferating in the neonatal mouse testis, which occurs until approximately pnd17 (Vergouwen et al., 1991). However, there was no increase in the proportion of tubules that were SOX9-positive between pnd5 and pnd8. This may be due to the higher proliferation rate in the germ cells, which would counteract the expected expansion in Sertoli cell number. The pieces of testis are not uniform in size or tubule composition, so quantification is given relative to tubule area and cannot detect absolute changes in number of cells. There were fewer Sertoli cells in the cultured tissue suggesting a loss of this cell type, but there were still a large number of these cells and they could still support the germ cell population. Unsurprisingly, the cultured tissue had substantially more cells undergo apoptosis than the fixed whole testes. However, the levels were still very low and appeared to have no effect on the testis structure.

In conclusion, the membrane culture using a basic media appeared suitable for culturing mouse postnatal testis tissue for four days and retained the majority of features of postnatal testis development, although there are areas for improvement such as ensuring an increase in germ cell numbers and maintaining the correct number of Sertoli cells. This would ensure the tissue was more representative of normal development.

Chapter 4. The effect of cyclophosphamide, cisplatin and doxorubicin on the prepubertal testis

4.1 Introduction

4.1.1 Chemotherapy and infertility

It was long believed that the testis was relatively protected from chemotherapy before puberty (Rivkees and Crawford, 1988). However, during prepubertal development there is germ cell division and Sertoli cell maturation, providing potential targets for chemotherapeutic drugs (Chemes, 2001), and children treated for cancer often suffer from infertility in adult life (Wallace et al., 2005). In order to develop preventative treatments, detailed information on how the testis is damaged and whether specific drugs cause injury through the same or different processes is required. There have been a number of studies examining the effect of different chemotherapy on the testis in rodents and these are discussed below for the three chemotherapeutic agents used in this chapter.

4.1.2 Cyclophosphamide

CYP is believed to be one of the most gonadotoxic anticancer agents. Exposure of adult male rats to CYP results in an increase in apoptosis of the germ cells (Cai et al., 1997). However, even after multiple doses, fertility recovered, with the majority of cell death occurring in the differentiating germ cells and the undifferentiated spermatogonia relatively protected (Drumond et al., 2011). As CYP must be activated in the liver the metabolite, PM has been used here to examine the effect of CYP on the prepubertal testis. There is currently no information on the direct effect of this metabolite on the testis.

4.1.3 Cisplatin

Studies in adult mice show damage to the seminiferous epithelium and increased apoptosis of germ cells (Köpf-Maier, 1992, Zhang et al., 2001, Seaman et al., 2003). It is the dividing spermatogonia that are most susceptible to CIS, suggesting that the loss of fertility may be reversible, as the stem cells survive (Huang et al., 1990). However, chemotherapeutic agents are given to patients in multiple cycles and therefore the response to an initial exposure may affect the level of damage caused by later exposures. A multi-cycle exposure model was developed (Sawhney et al., 2005) and it showed increased proliferation following exposure to CIS (Harman and Richburg, 2014). This made the testis even more susceptible to CIS induced damage on the second exposure, potentially targeting the SSCs, leading to irreversible azoospermia (Harman and Richburg, 2014).

4.1.4 Doxorubicin

DOX is considered to be the least gonadotoxic of the agents examined here. However, mice exposed to DOX prepuberty experienced apoptosis of germ cells (Brilhante et al., 2011), and it has been shown that the initiation of spermatogenesis may be particularly vulnerable (Hou et al., 2005). There is evidence of functional damage to Sertoli cells, but this was secondary to germ cell loss (Brilhante et al., 2012). One *in vitro* investigation of DOX induced testicular damage using an organ culture of pnd5 testis (Nurmio et al., 2009b), similar to that developed here. This showed a loss of germ cells without effect on the Sertoli cell population. However, only a very short time window could be examined due to control tissue showing high levels of cell death and loss of tissue integrity (Nurmio et al., 2009b).

4.1.5 Summary of previous studies

In all the studies above the primary mechanism of damage to the testis was through loss of germ cells. The studies mentioned above for CYP and CIS were performed using adult mice or rats. It cannot be assumed that these are applicable to the prepubertal testis, which has a different pattern of germ and somatic cell division. Therefore, it is important to examine the effect of chemotherapy before puberty. The *in vitro* model used here also means the direct effect of the drugs can be examined, the amount of chemotherapy reaching the testis is controlled, and young mice are not exposed to toxic chemotherapy drugs.

4.1.6 Aims

The aims of the chapter were:

- To examine the effect of three chemotherapeutic agents; CYP, CIS and DOX on the cell populations of the prepubertal mouse testis using an *in vitro* culture system.
- To examine whether proliferation or apoptosis is affected by exposure to CYP, CIS or DOX.

4.2 Methods

4.2.1 Culture of postnatal mouse testis tissue

A minimum of two pnd5 C57Bl/J6 mouse testes per culture were dissected (see 2.1.1 and 2.1.2). Each piece was transferred into a 24 well plate, on a Whatman Nucleopore membrane floating on 1 ml of basic medium (see 2.2.1) and left for 24 h (Day 1). On Day 2 of culture, medium was supplemented with CIS, DOX or PM, each prepared as described (see 2.3). Media were replaced half in—half out to produce final concentrations (Table 4.1). Four doses of PM were selected due to the wide range of reported serum levels in patients (Tasso et al., 1992, Struck et al., 1987). On Day 3, membranes were transferred into fresh control medium. For the final

day in culture (Day 4), medium was additionally supplemented with 15 $\mu\text{g ml}^{-1}$ 5-bromo-2'-deoxyuridine (BrdU; Sigma Aldrich Ltd, UK). After 24 h, tissue was fixed and processed as described (see 2.4). For each chemotherapeutic agent a minimum of 7 testis fragments from each concentration were examined. These fragments came from a minimum of 3 cultures, except for 1 $\mu\text{g ml}^{-1}$ PM where fragments were taken from 2 cultures only.

Table 4.1 Final concentrations of chemotherapeutic agents

Concentration	PM	CIS	DOX
Low	0.01 μM	0.1 $\mu\text{g ml}^{-1}$	0.05 $\mu\text{g ml}^{-1}$
Mid	0.1 μM 1 μM	0.5 $\mu\text{g ml}^{-1}$	0.1 $\mu\text{g ml}^{-1}$
High	10 μM	1 $\mu\text{g ml}^{-1}$	0.5 $\mu\text{g ml}^{-1}$

4.2.2 Haematoxylin and eosin staining

H&E staining was performed as described (see 2.5).

4.2.3 Immunohistochemistry

Immunohistochemistry was performed as described (see 2.6) with the antibody combinations as detailed in Table 4.2.

Table 4.2 Antibody combinations for immunochemistry

Primary I	Dilution	Secondary I	Detection	Primary 2	Dilution	Secondary 2	Retrieval
BrdU	1:500	G α -rat Bio	Strep 488nm	MVH	1:100	G α -M 568nm	Yes
SOX9	1:500	G α -R Bio	Strep 488nm	BrdU	1:500	G α -rat 568nm	Yes
SOX9	1:500	G α -R Bio	Strep 488nm	N/A	N/A	N/A	No
COUPTF II	1:200	G α -M Bio	Strep 568nm	CC3	1:500	G α -R 488nm	Yes
CC3	1:500	G α -R Bio	Strep 488nm	MVH	1:100	G α -M 568nm	Yes

G α -rat Bio = Goat anti rat Biotinylated

G α -rat 568nm = Goat anti ratIgG₁ 568n

4.2.4 Image acquisition and analysis

Images were acquired and analysed as described (see 2.7 and 2.8).

4.2.5 Statistical analysis

Data were analysed using GraphPad Prism. To determine statistical significance between control and different concentrations of each drug, one-way ANOVA was performed followed by a Dunnett's *post hoc* test, if the ANOVA was significant. Where the data were not normally distributed a Kruskal-Wallis test was performed followed by Dunn's multiple comparison tests. All results are given as mean \pm SEM with statistical significance set at $p < 0.05$.

4.3 Results

Firstly, the effect of PM on the neonatal mouse testis was examined as CYP is thought to be one of the most gonadotoxic chemotherapeutic agents and therefore it was used to test if the *in vitro* system was able to produce the same results *in vivo*. Next two other drugs, CIS and DOX were examined. These show differing effects in the ovary with CIS targeting the germ cells and DOX primarily targeting the supporting granulosa cells (Morgan et al., 2013).

4.3.1 Phosphoramidate mustard

4.3.1.1 Depletion of germ cells at the highest doses of phosphoramidate mustard

In the control testis, MVH-positive germ cells were situated predominantly along the basement membrane of the seminiferous tubules and the majority were BrdU-positive (Fig. 4.1A). In response to exposure to PM there was a loss of the germ cells from the seminiferous tubules in a concentration dependent manner (Fig. 4.1B–E). At the two highest doses of PM there was a significant decrease in the proportion of germ cell area to tubule area, with none observed at the highest dose (Fig. 4.1F).

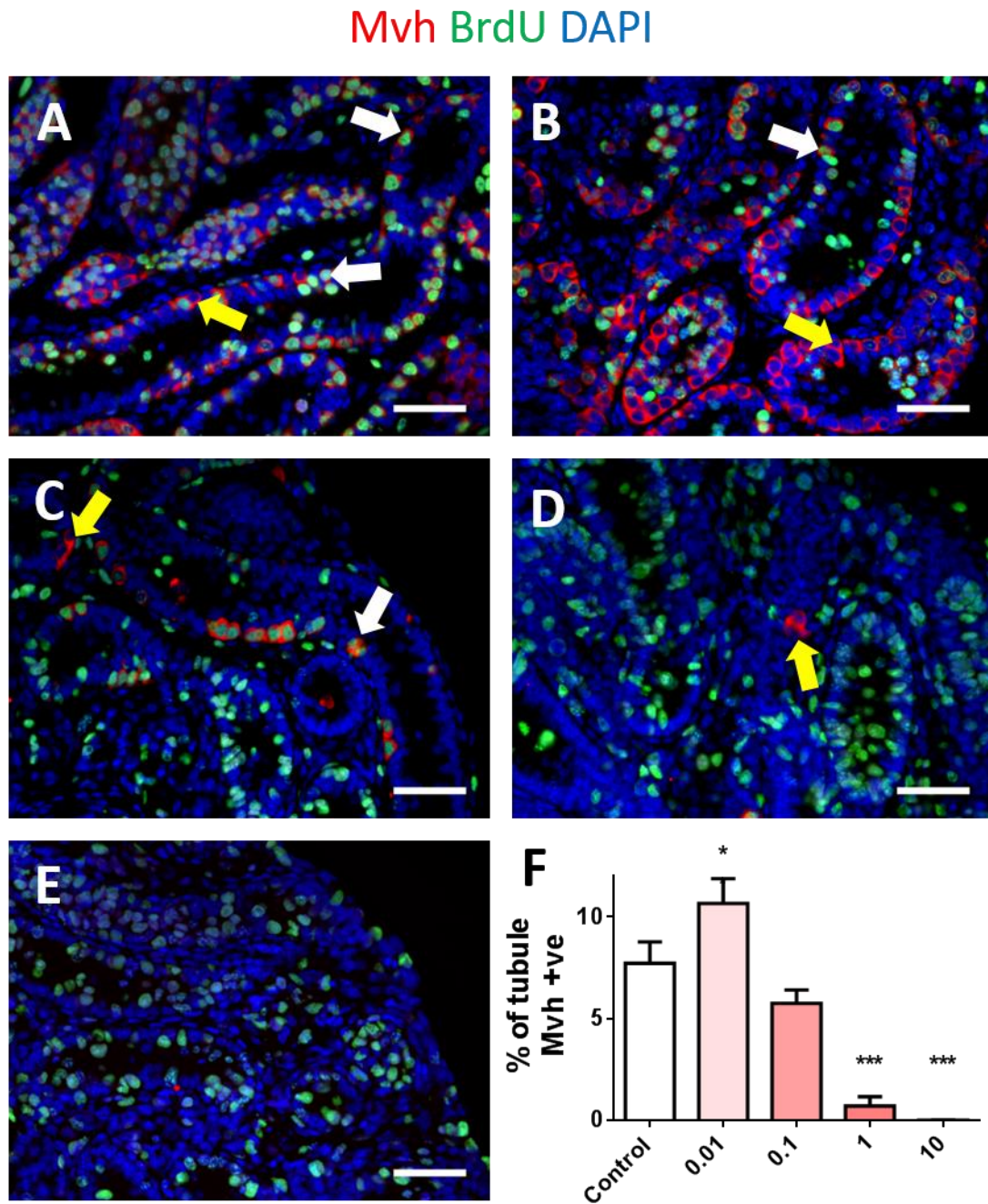


Figure 4.1 Germ cells and proliferation on exposure to PM. A-E) Immunohistochemical localisation of MVH (red), BrdU (green), counterstained with DAPI (blue) for A) control cultured testis and fragments exposed to B) 0.01 μM , C) 0.1 μM , D) 1 μM , and E) 10 μM PM. White arrows indicate germ cells that are also BrdU-positive. Yellow arrows indicate germ cells without BrdU. Scale bars = 50 μm . F) The percentage of tubule area containing MVH-positive germ cells was quantified with $n=8$ for each concentration. One-way ANOVA followed by Dunnet's *post hoc* tests were applied. Data are mean \pm SEM. * $p < 0.01$, *** $p < 0.001$ for each concentration of PM versus control.

4.3.1.2 Lack of effect of phosphoramidate mustard on somatic cells

The somatic cells, Sertoli and interstitial cells, were examined to see if they were affected by PM. The SOX9-positive Sertoli cells were found lining the seminiferous tubules in the control and some of these cells were proliferating, which is expected in early postnatal life (Fig. 4.2A). Even at the highest dose of PM, Sertoli cells were still present throughout the tubules and were dividing (Fig. 4.2B-E). There was no significant effect on the proportion of the tubule area occupied by Sertoli cells on exposure to PM ($p = 0.38$; Fig. 4.2F).

Interstitial cells were examined by determining expression of COUPTFII, which is expressed in the majority of the interstitial cells in the neonatal mouse. It also labels the ALSCs that will differentiate in later life (Kilcoyne et al., 2014). The interstitium contained mainly COUPTFII-positive cells both in the control cultured testis and at the every concentration of PM (Fig. 4.3A-E). There was a trend towards an increase in the percentage of the interstitial area that contained COUPTFII-positive interstitial cells on exposure to PM, but this did not reach significance ($p = 0.07$; Fig. 4.3F).

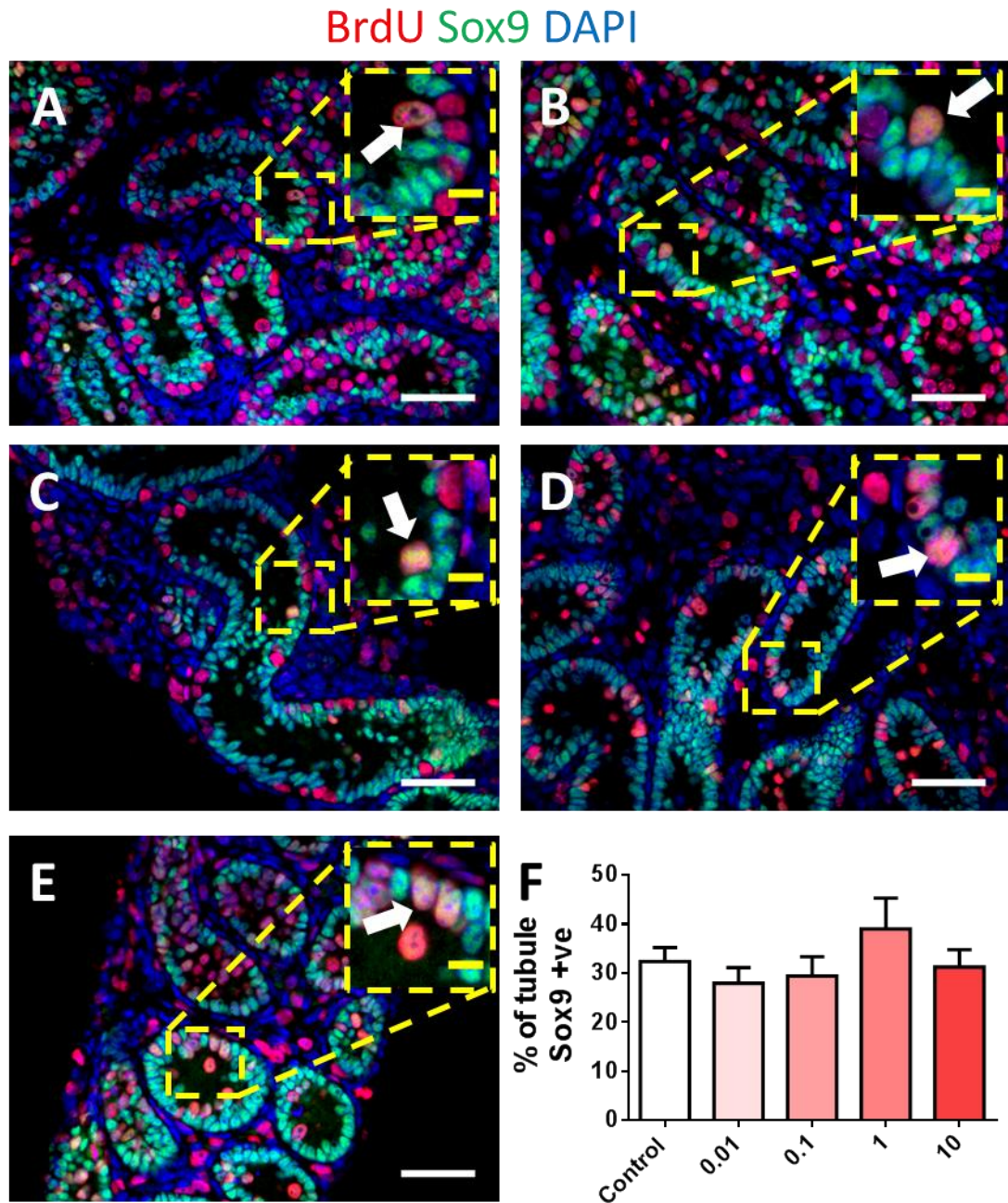


Figure 4.2 Sertoli cells on exposure to PM. A-E) Immunohistochemical localisation of SOX9 (green), BrdU (green), counterstained with DAPI (blue) for A) control cultured testis and fragments exposed to B) 0.01 μM, C) 0.1 μM, D) 1 μM, and E) 10 μM PM. Scale bars = 50 μm. F) The percentage of tubule area containing SOX9-positive cells was quantified with n = 9 for the control tissue, n = 8 for 0.01 μM and 1 μM, and n = 7 for 0.1 μM and 10 μM. One way ANOVA was applied but was not significant. Data are mean ± SEM.

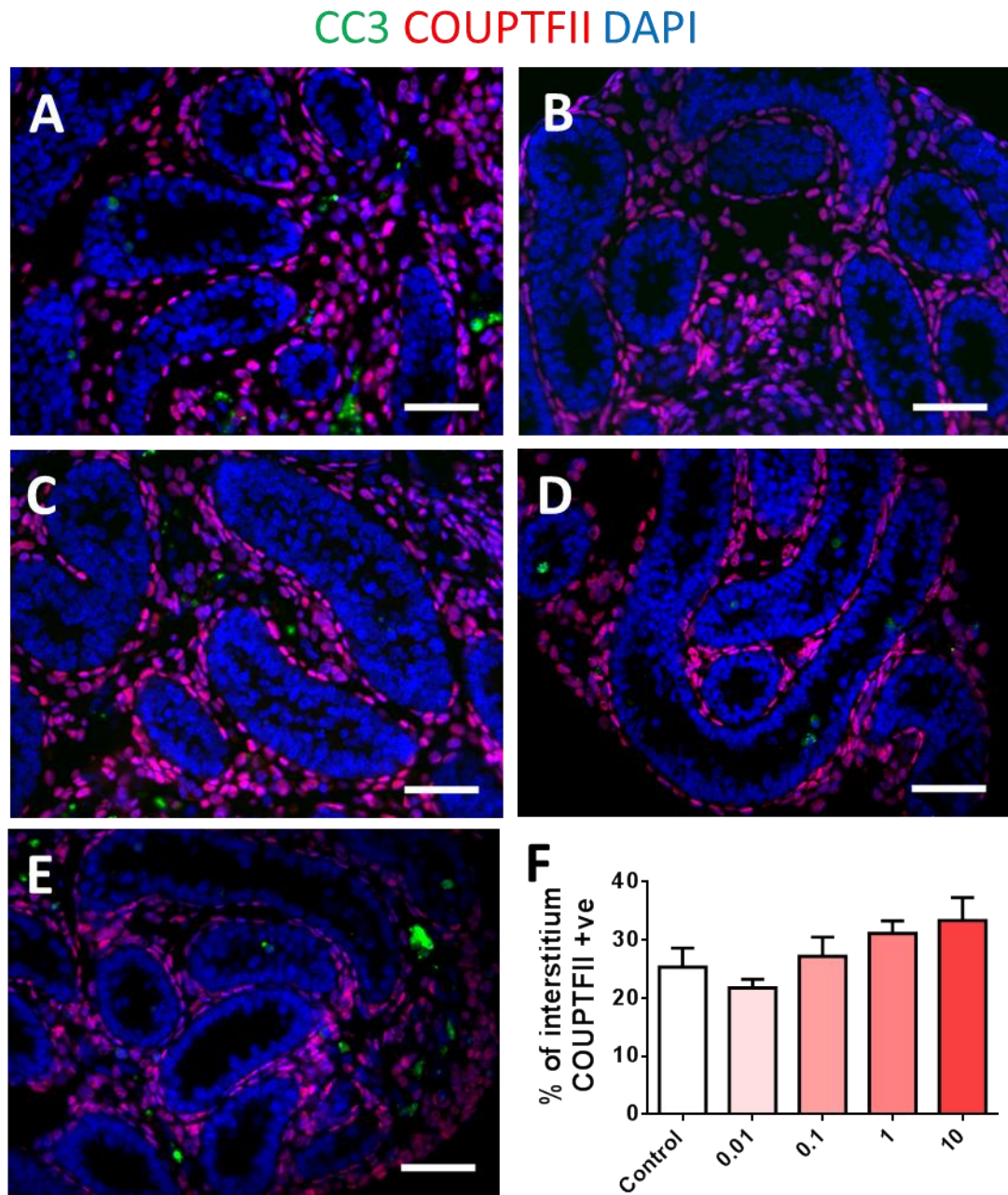


Figure 4.3 Interstitial cells and apoptosis on exposure to PM. A-E) Immunohistochemical localisation of COUPTFII (red), CC3 (green), counterstained with DAPI (blue) for A) control cultured testis and fragments exposed to B) 0.01 μM, C) 0.1 μM, D) 1 μM, and E) 10 μM PM. Scale bars = 50 μm. F) The percentage of the interstitial area containing COUPTFII-positive cells was quantified with $n = 8$ for each concentration, except 10 μM where $n = 7$. One-way ANOVA was applied but did not reach statistical significance. Data are mean \pm SEM.

4.3.1.3 Lack of effect of phosphoramidate mustard on proliferation

BrdU was added to the culture for the final 24 h, in order to examine the proliferative capacity of the tissue following exposure to PM. There was no effect on the percentage of the section area that was BrdU-positive at any concentration of PM ($p=0.43$; Fig. 4.4A). In the controls approximately 70% of the proliferation occurred in the tubules. This decreased to 50% in the exposed tissue, however, the decrease did not reach significance ($p=0.10$; Fig. 4.4B).

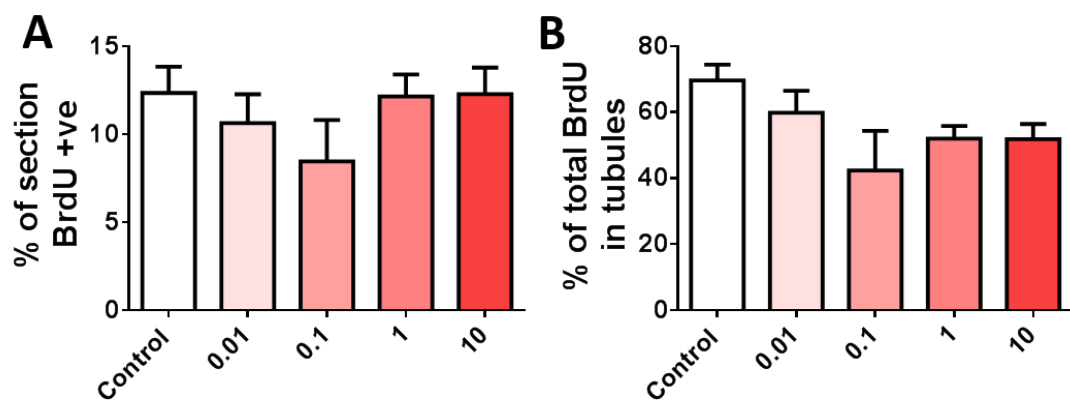


Figure 4.4 Quantification of proliferation on exposure to PM. A) The percentage of the testis fragment area that was BrdU-positive was quantified for each concentration of PM. B) The proportion of total BrdU that was found within the tubules with $n=8$ testis fragments from each concentration. One-way ANOVA were applied but did not reach significance. Data are mean \pm SEM.

4.3.1.5 Increase in apoptosis at the highest dose of phosphoramidate mustard

Apoptosis was examined by carrying out immunohistochemistry for CC3. There was very little CC3 at the end of culture in both control and exposed sections (Fig. 4.3A–E). The majority of CC3 was found in the interstitium (Fig. 4.5A). This was unexpected as CC3 would be anticipated to be high in the tubules due to the specific loss of germ cells. Despite this, CC3 expression increased threefold at the highest dose of PM in comparison to control cultured testis (Fig. 4.5B). This pattern was replicated when CC3 was just examined within the tubules (Fig. 4.5C), except at 0.1 μ M, where a single piece showing very high levels of CC3 skewed results somewhat.

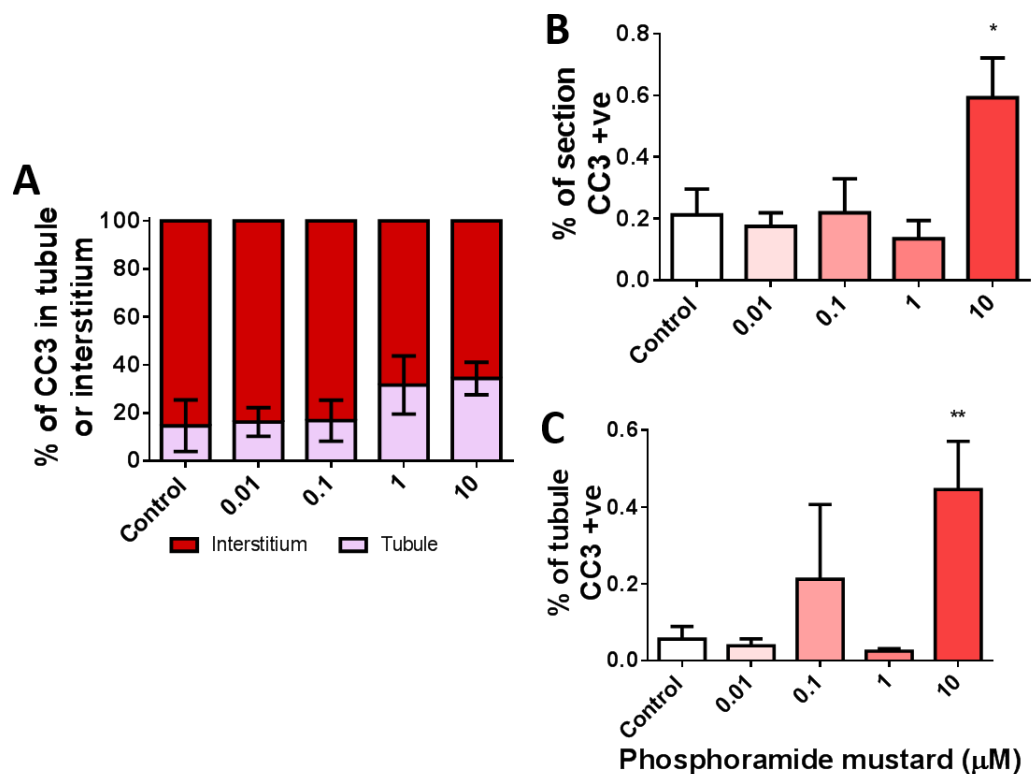


Figure 4.5 Quantification of apoptosis on exposure to PM. A) The proportion of CC3 in each testis compartment, tubules or interstitium was quantified for each concentration of PM along with the percentage of B) total section area or C) tubule area that is CC3-positive for each concentration. $n = 8$ for each concentration, except $10 \mu\text{M}$ where $n = 7$. The level of CC3 was not normally distributed and therefore a Kruskal-Wallis test was applied followed by Dunn's *post hoc* test. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ for PM versus control.

4.3.2 Cisplatin and doxorubicin

4.3.2.1 Structure of testis following exposure to cisplatin or doxorubicin

Examination of H&E sections showed that both the control tissue and samples exposed to CIS or DOX had morphologically normal tubules with basement membrane separating the tubules from the interstitium. At the lowest dose of CIS or DOX a few germ cells could be observed either at the basement membrane or in the centre of the tubules (Fig. 4.6). At the high dose of CIS or DOX germ cells could not be identified and there were many pyknotic cells. As the concentration of CIS or DOX increased there was a reduction in the tubule area to section area ratio and a reduction in the tubule diameter (Fig. 4.7)

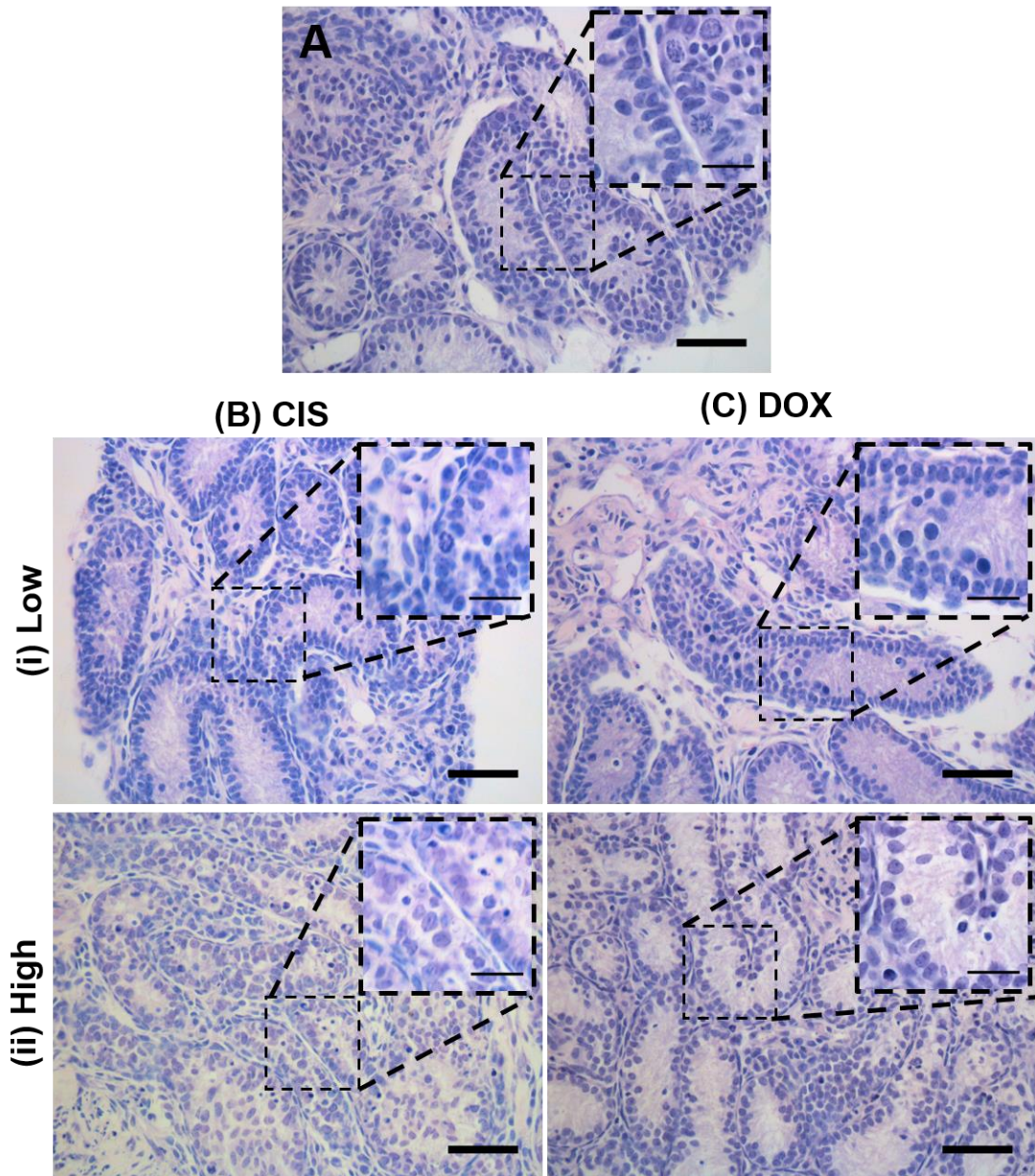


Figure 4.6 H&E of postnatal mouse testis tissue on exposure to CIS or DOX. A) Control cultured testis and fragments exposed to B) CIS or C) DOX at the (i) lowest and (ii) highest concentration. Main image scale bars = 50 μ m, inset scale bars = 20 μ m.

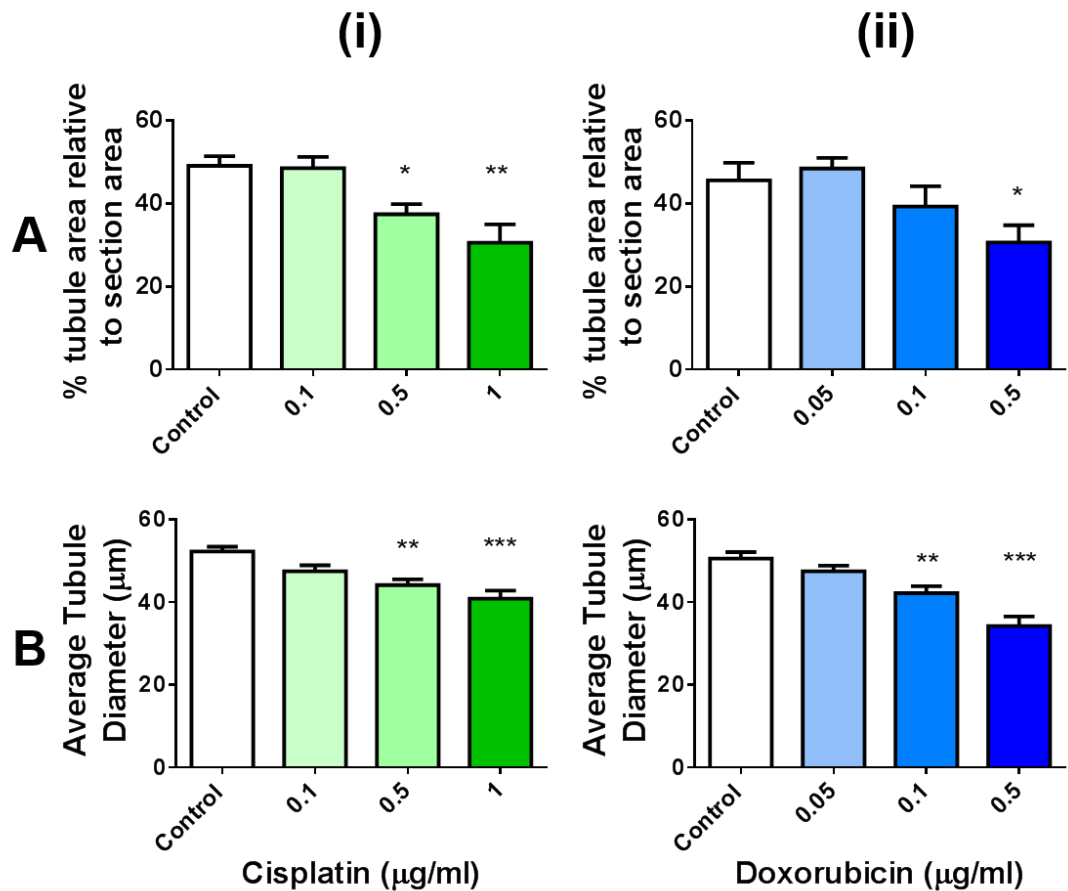


Figure 4.7 Quantification of tubule properties on exposure to CIS or DOX. Quantification of A) proportion of tubule area to section area, and B) tubule diameter with $n = 8$ for each concentration of (i) CIS or (ii) DOX. One-way ANOVA followed by Dunnet's *post hoc* tests were applied. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for CIS or DOX versus control.

4.3.2.2 Loss of germ cells following exposure to cisplatin or doxorubicin

In the control cultures the majority of germ cells were BrdU-positive (Fig. 4.8A). In response to exposure to CIS or DOX there was a significant loss of germ cells from the seminiferous tubules (Fig. 4.8B, Fig. 4.8C); this occurred at even the lowest concentrations of CIS or DOX (Fig. 4.8Bi, Fig. 4.8Ci). However, even at the highest dose of CIS or DOX there were examples of germ cells that had survived exposure. Some of these remaining germ cells were BrdU-positive (Fig 4.8Bii).

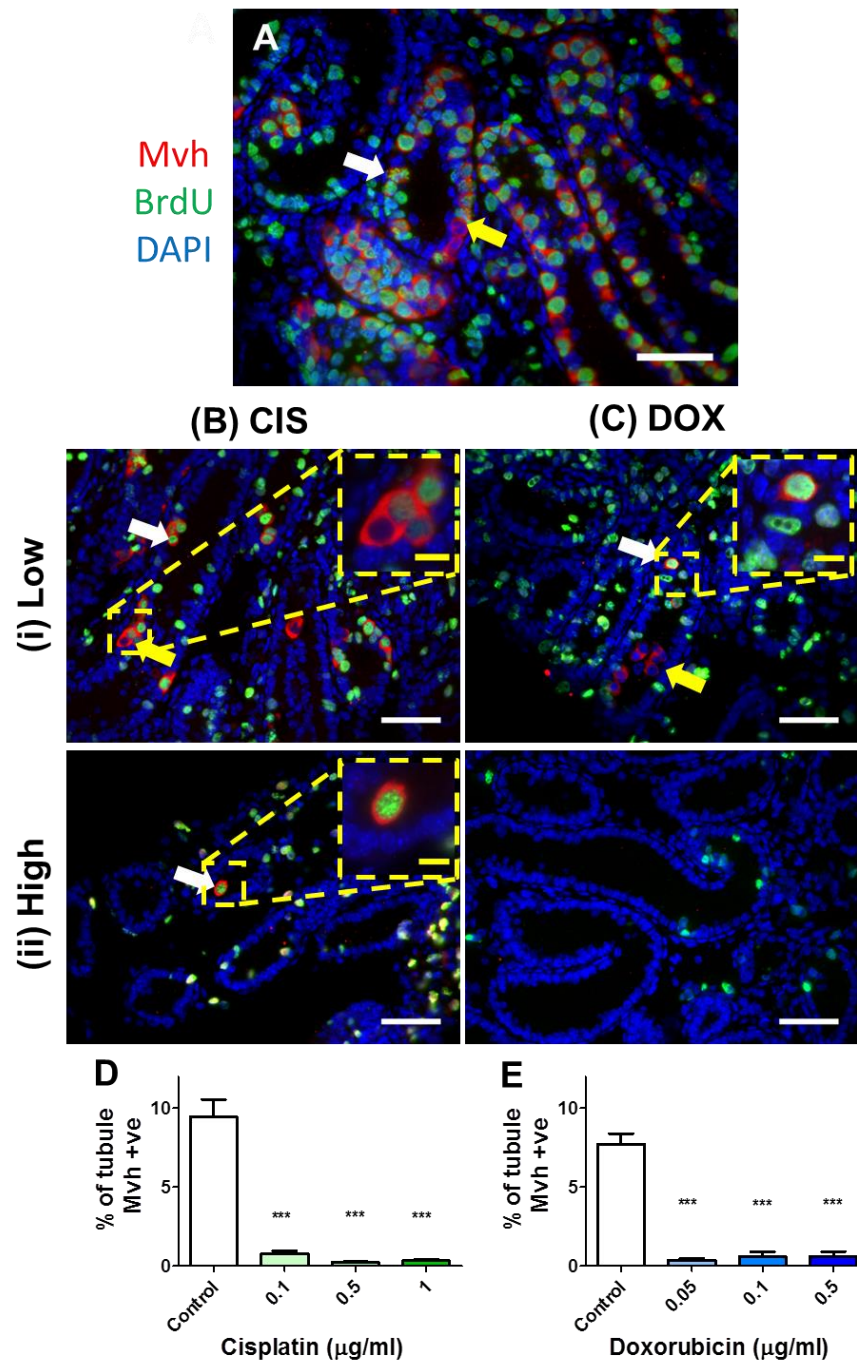


Figure 4.8 Germ cells and proliferation on exposure to CIS or DOX. A-C) Immunohistochemical localisation of MVH (red), BrdU (green), counterstained with DAPI (blue) for A) control cultured testis and fragments exposed to B) CIS and C) DOX at the (i) lowest and (ii) highest concentration. White arrows indicate germ cells that are also BrdU-positive. Yellow arrows indicate germ cells without BrdU. White scale bars = 50 μm , yellow scale bars = 10 μm . The percentage of tubule containing MVH-positive germ cells for D) CIS or E) DOX was quantified with $n = 8$ for each concentration. One-way ANOVA followed by Dunnet's *post hoc* tests were applied. Data are mean \pm SEM. *** $p < 0.001$ for CIS or DOX versus control.

4.3.2.3 *Lack of effect of cisplatin or doxorubicin on somatic cells*

Somatic cells were examined to see if they were affected by CIS or DOX. In contrast to the loss of germ cells, on exposure to the highest concentration of CIS and DOX the Sertoli cells were still present throughout the tubules and were dividing (Fig. 4.9). There was no significant effect on the proportion of the tubule area occupied by Sertoli cells on exposure to either CIS ($p = 0.11$), or DOX ($p = 0.45$; Fig. 4.9D–E).

The interstitium contained mainly COUPTFII-positive cells in the control cultured testis and at the highest concentration of CIS and DOX (Figure 4.10A–C) There was no effect of drug on the percentage of the interstitial area that contained COUPTFII-positive interstitial cells on exposure to CIS ($p = 0.86$), or DOX ($p = 0.34$; Fig. 4.10D–E).

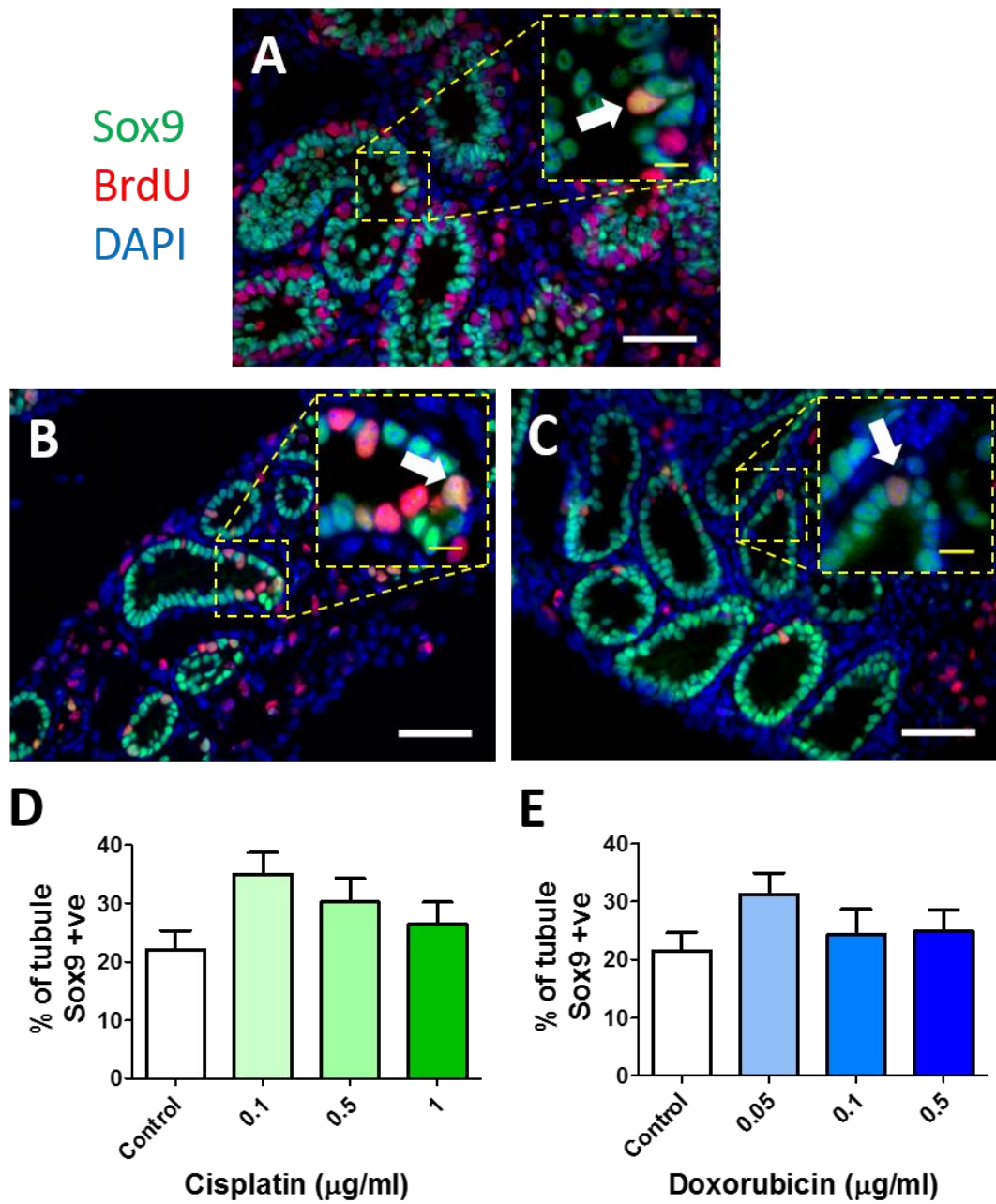


Figure 4.9 Sertoli cells on exposure to CIS or DOX. A-C) Immunohistochemical localisation of SOX9 (green), BrdU (green), counterstained with DAPI (blue) for A) control cultured testis, and fragments exposed to the highest concentration of B) CIS and C) DOX. White arrows indicate cells co-staining for SOX9 and BrdU. Main image scale bars = 50 µm, yellow scale bars in insets = 10 µm. The percentage of tubule area containing SOX9-positive cells for D) CIS or E) DOX was quantified with $n = 8$ for each concentration. One-way ANOVA were applied but were not significant. Data are mean \pm SEM.

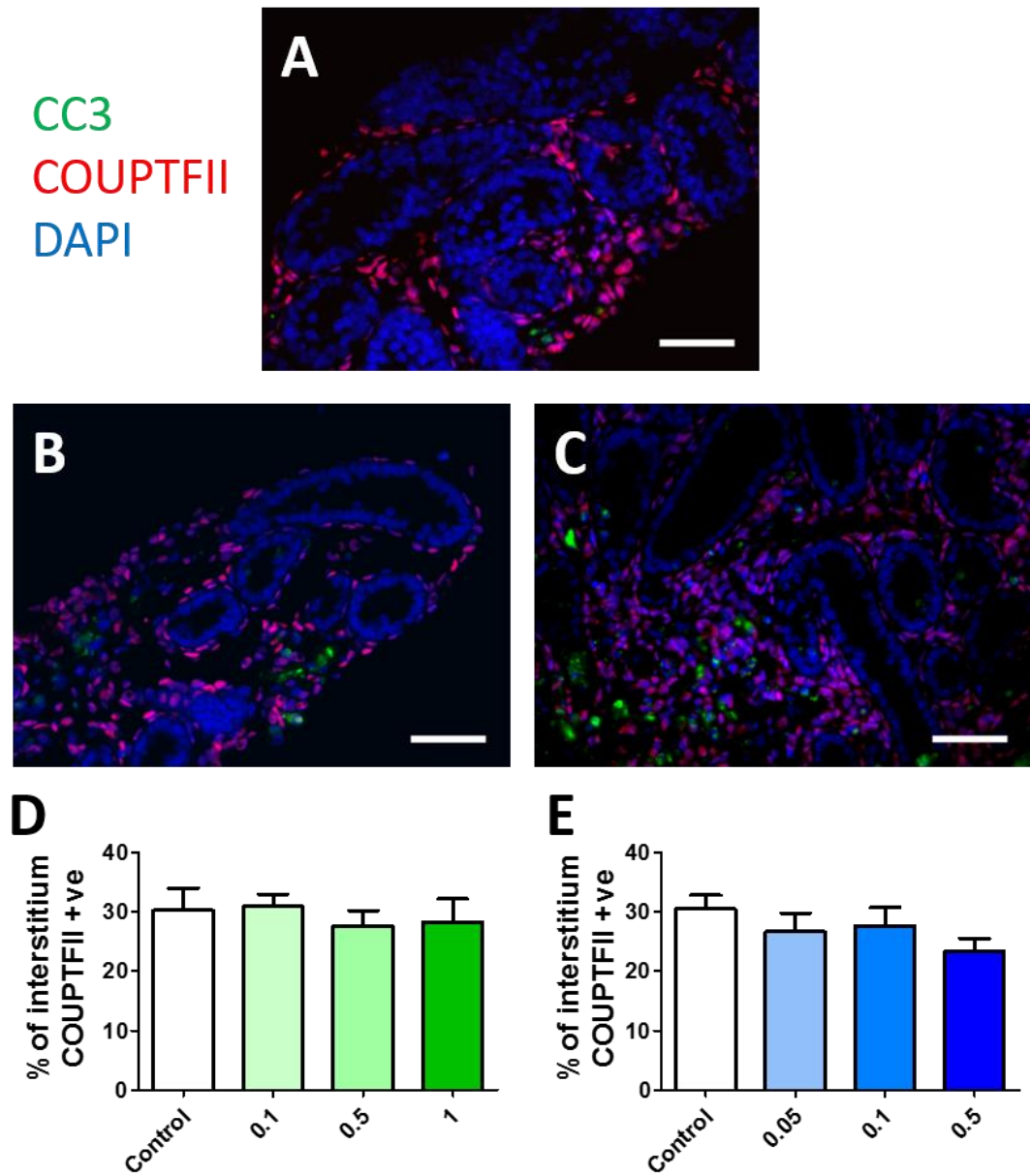


Figure 4.10 Interstitial cells and apoptosis on exposure to CIS or DOX. A-C) Immunohistochemical localisation of COUPTFII (red), CC3 (green), counterstained with DAPI (blue) for A) control cultured testis, and fragments exposed to the highest concentration of B) CIS or C) DOX. Scale bars = 50 μ m. The percentage of the interstitial area containing COUPTFII-positive cells for D) CIS or E) DOX was quantified with $n = 8$ for each concentration. One-way ANOVA were applied but were not significant. Data are mean \pm SEM.

4.3.2.4 Decrease in proliferation following exposure to cisplatin or doxorubicin

Proliferation was examined following BrdU administration for the final 24 h of culture. The total amount of BrdU significantly decreased at all concentrations of CIS or DOX (Fig. 4.11A). When this proliferation was investigated separately for the tubule and interstitial areas, a significant decrease in the percentage of the tubule area that was BrdU-positive was observed at all concentrations of CIS or DOX (Fig. 4.11B). There was a trend towards a decrease in proliferation in the interstitium, but this only reached significance at the highest dose of DOX (Fig. 4.11C). In the control cultured testis, the majority of BrdU was found within the tubules (Fig. 4.11D), but this decreased at all concentrations on exposure to CIS or DOX, reflecting the loss of dividing germ cells.

4.3.2.5 Increase in apoptosis following exposure to cisplatin or doxorubicin

There was very little CC3 at the end of culture in all sections (Fig. 4.10A–C). The majority was found in the interstitium (Fig. 4.12A), rather than in the tubules, as would have been expected due to the specific loss of germ cells. Despite this, CC3 expression increased 3.5- and 5.2-fold at the highest dose of CIS and DOX respectively, in comparison to control cultured testis (Fig. 4.12B). This pattern was replicated when CC3 was examined just within the tubules (Fig. 4.12C; 5.2- and 6.5-fold increase).

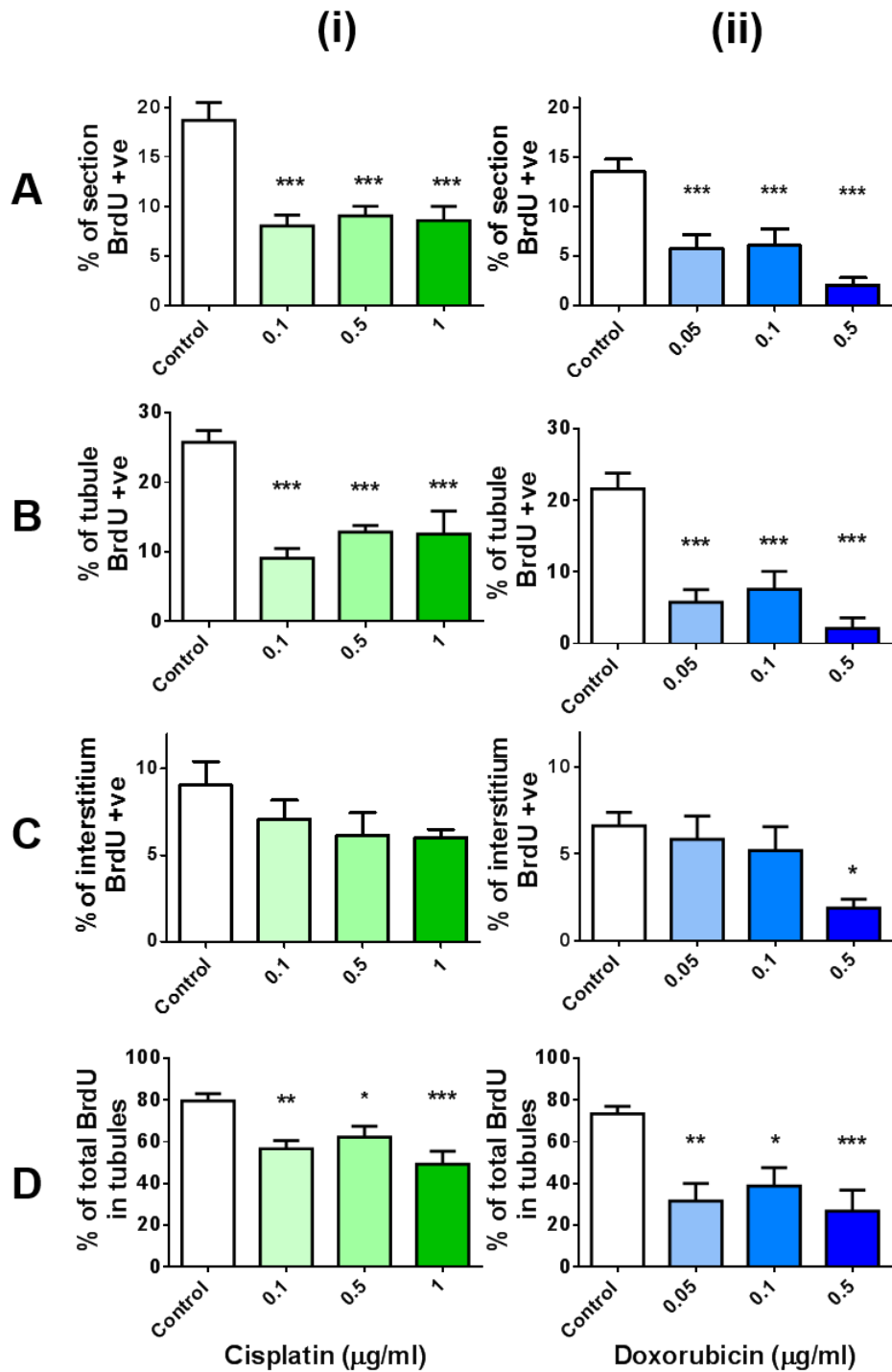


Figure 4.11 Quantification of proliferation on exposure to CIS or DOX. A) The percentage of the testis that was BrdU-positive was quantified for each concentration of (i) CIS or (ii) DOX. This was split into B) percentage of tubule area that was BrdU-positive, and C) percentage of the interstitium area that was BrdU-positive. D) The proportion of total BrdU that was found within the tubules with $n = 8$ for each concentration. One-way ANOVA followed by Dunnett's *post hoc* tests were applied. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for CIS or DOX versus control.

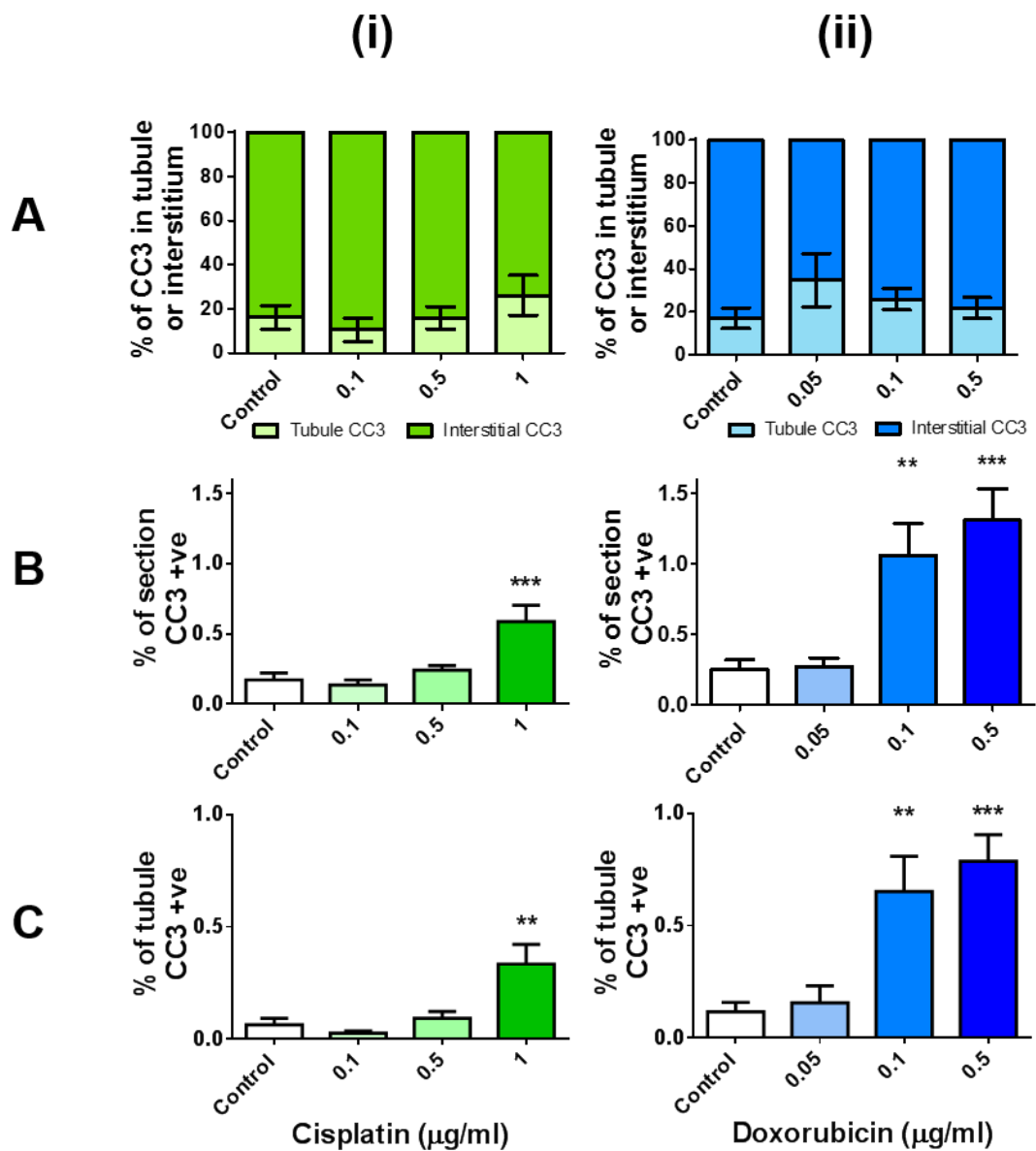


Figure 4.12 Quantification of apoptosis on exposure to CIS or DOX. A) The proportion of CC3 in each testis compartment, tubules or interstitium was quantified along with the percentage of B) total section area or C) tubule area that is CC3-positive for each concentration of (i) CIS or (ii) DOX with $n = 8$ testis fragments from each concentration. One-way ANOVA followed by Dunnet's *post hoc* tests were applied. Data are mean \pm SEM ** $p < 0.01$, *** $p < 0.001$ for CIS or DOX versus control.

4.3.2.6 *Effect of cisplatin or doxorubicin after only 24 hours in exposure*

As all of the germ cells were depleted by the end of the 4 day culture protocol at all concentrations of CIS or DOX, some tissue was fixed 24 h after the addition of each of the chemotherapeutic agents, rather than transferring into fresh media. The control tissue that had not been exposed to either chemotherapeutic agent showed many MVH-positive germ cells and little apoptosis in both the tubules and interstitium (Fig. 4.13A). At the lowest dose of either CIS or DOX there appeared to be fewer germ cells (Fig. 4.13Bi, Fig. 4.13Ci), and at the highest dose of CIS there was a reduced number of germ cells, but with many still surviving (Fig. 4.13Bii). At the highest dose of DOX nearly all the germ cells had already been depleted and there was a very high level of CC3 expression (Fig. 4.13Cii).

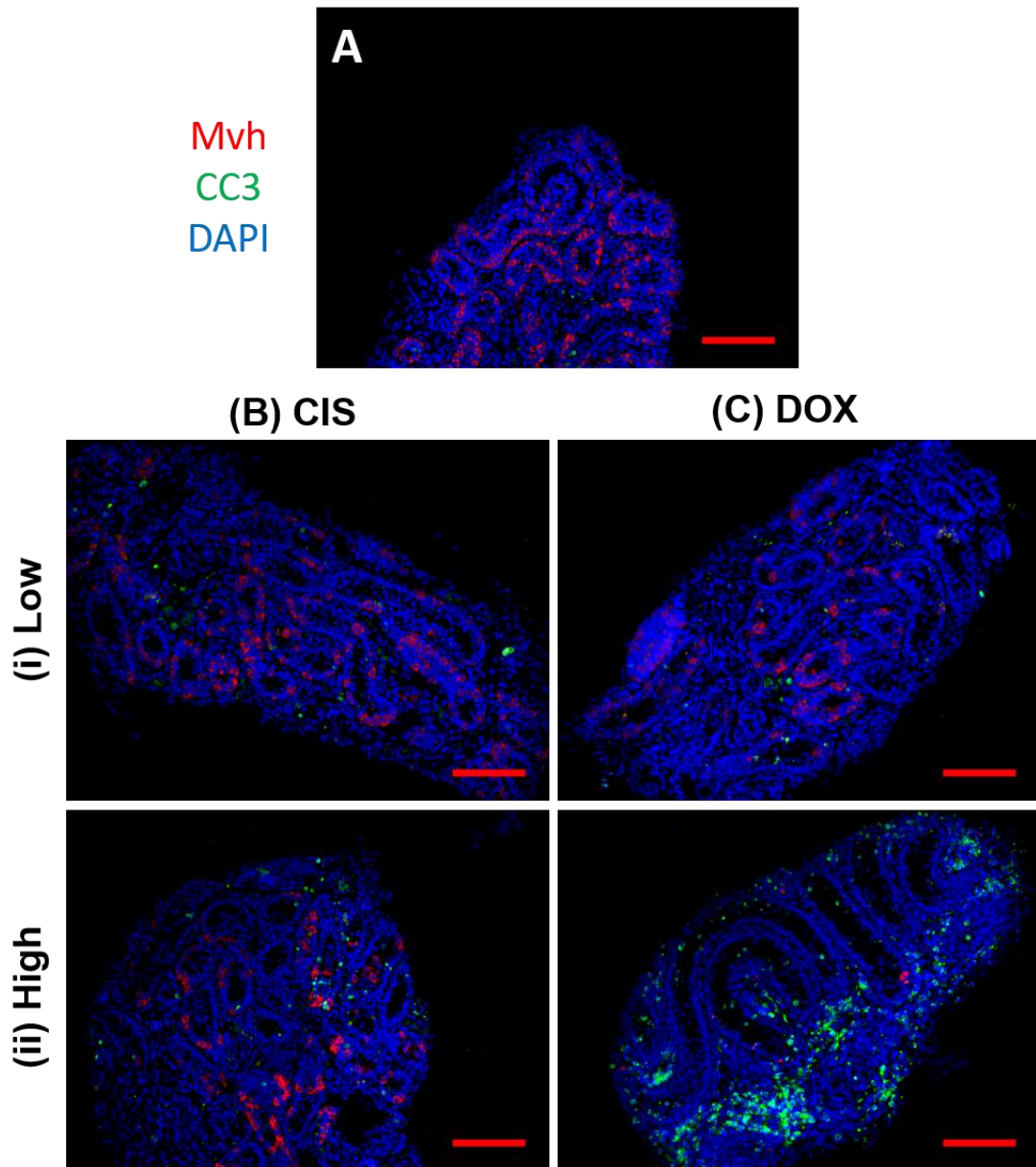


Figure 4.13 Germ cell and apoptosis after 24 hours exposure to CIS or DOX. Immunohistochemical localisation of MVH (red) and CC3 (green), counterstained with DAPI (blue). A) Control cultured testis and fragments exposed to B) CIS or C) DOX and fixed after 24 hours exposure at the (i) lowest and (ii) highest concentration. Images are representative of a single culture with only one or two cultured fragments per concentration. Scale bars = 100 μm .

4.4 Discussion

The results show a loss of germ cells when postnatal mouse testes were exposed to PM, CIS or DOX. The doses used here were chosen to be within the range of patients' serum which is 0.1–10 μM PM (Tasso et al., 1992, Struck et al., 1987), 0.4–1 $\mu\text{g ml}^{-1}$ CIS (Urien and Lokiec, 2004, Crom et al., 1981) and 0.01–0.6 $\mu\text{g ml}^{-1}$ DOX (Greene et al., 1983, Gewirtz, 1999). As most chemotherapy drugs primarily target dividing cells, the loss of germ cells is expected, as these are proliferating in the neonatal testis. This also fits with previous studies on rodents exposed to CYP, CIS or DOX, which show primarily a loss of the germ cells from the testis. However, even when the majority of germ cells were depleted there were a number that survived, and these were often dividing. If any of these cells were SSCs then over a long time period these could re-colonise the seminiferous tubules and contribute to recovery of fertility. Of all the germ cells, it would be expected that the stem cells would survive as they have a slower cell cycle. It has also been shown that in adult mice the differentiating spermatogonia are the most sensitive to chemotherapy induced damage and that the stem population is relatively protected (Drumond et al., 2011).

All three chemotherapeutic agents had no effect on the number of Sertoli cells and interstitial cells. This supports previous studies indicating Sertoli cell number was not altered on exposure to CIS (Sawhney et al., 2005). However, it has been suggested that other properties may be altered, such as Sertoli cell shape and connections between cells (Köpf-Maier, 1992). Therefore Sertoli cell function and maturation is worth investigating. Over the course of neonatal development the Sertoli cells change from actively proliferating immature Sertoli cells expressing markers such as AMH, to the more mature quiescent cells expressing the AR (Sharpe et al., 2003). These markers could be examined to see if the Sertoli cells are maturing correctly or if maturation was altered by exposure to any of the

chemotherapeutic agents. Sertoli cells are vital for forming the SSC niche by secreting a wide variety of signalling molecules. One of the most important is GDNF (Oatley and Brinster, 2012). GDNF increased following exposure to multiple cycles of CIS (Harman and Richburg, 2014) and it would therefore be interesting to see if this was affected by CIS, DOX or PM.

BrdU was added to the media for all of the final 24 h of culture and therefore marks all proliferation during this period. Proliferation was significantly decreased following exposure to all concentrations of CIS or DOX, but not in sections exposed to PM. This was surprising as at the highest dose of PM there was a loss of the germ cells, which are dividing in the control tissue. Therefore, with this population being lost, it would be expected the total proliferation would decrease. The fact there is no decrease would suggest that the surviving cells have increased proliferation. At the highest dose of DOX there was also a decrease in proliferation in the interstitium with very few cells dividing. This could indicate effects beyond the loss of germ cells. There was a downward trend, but not a significant decrease, in the number of COUPTFII-positive cells in the interstitium. If the ALSCs were lost then this could result in hypogonadism in later life.

There was little apoptosis in both control and treated sections, however, when CYP is given to adult rats, apoptosis returns to control levels after 48 h (Cai et al., 1997) and therefore the wave of apoptosis may have been missed. The cell death was not confined to the tubules with the majority located within the interstitium by the end of the 4 day culture. This indicates that there are changes to the interstitium following exposure to chemotherapeutic agents, even if this could not be detected by the total expression of COUPTFII in the interstitium, which was not significant in any of the three chemotherapeutic agents. Other, more specific markers of different interstitial cell types such as 3β -HSD for Leydig cells, smooth muscle actin for the

PTMs and endothelial cells could be examined along with testosterone production by the cultured tissue, to be able to get a more complete picture of any damage to the interstitium.

After 24 h the two drugs appeared to have different effects with a large amount of apoptosis at the high concentration of DOX with almost complete depletion of the germ cells. Conversely in the CIS treated tissue there was still many germ cells remaining. These must have been destined to die in the next 48 h as at the end of the 4 day culture there were very few germ cells remaining. This may indicate that the different drugs have different mechanisms of action or that the time taken to initiate the same mechanism may vary between drugs. The initial damage could be investigated by carrying out a time course analysis and this is discussed further in **6.2.2**. At the concentrations studied here all three chemotherapeutics had similar actions. It was surprising that they all acted the same, as they are believed to have different gonadal toxicities, with CYP being associated with permanent azoospermia and CIS and DOX reversible azoospermia (Meistrich, 2013). In fact, at the doses used here CIS and DOX appeared more gonadotoxic. Doses were based on patients' serum level, which is highly variable both between patients and between studies. The three drugs may have a different distribution throughout the body and therefore different concentrations to the ones presented here may reach the testis in clinical practice. More information is required on the levels of chemotherapeutics in the testis in children treated for cancer to inform scientific studies.

All three chemotherapy drugs were all given as a single chemotherapeutic agent, whereas in clinical practice patients are not exposed to a single drug but a combination of many drugs, which may have additive or synergistic effects.

In conclusion, all three chemotherapeutic drugs examined here appeared to have very similar effects on the testis causing a specific loss of germ cells without effect on the somatic cells.

Chapter 5. Human foetal testis culture

5.1 Introduction

5.1.1 Culture of human testis tissue

Efforts to culture human testicular tissue date back to the mid-20th century, when it was first proposed as a method for *in vitro* spermatogenesis (Steinberger, 1975). Adult human testis cultures have mainly focused on the ability of the tissue to enter meiosis and proved unsuccessful (Roulet et al., 2006). Subsequently, testis culture has been utilised to study the effects of exogenous agents such as phthalates (Desdoits-Lethimonier et al., 2012) and analgesics (Albert et al., 2013) on the testis.

The culture of human foetal testis has been used to examine the effects of a wide variety of compounds (Habert et al., 2014). Results appear mixed, with some reporting preservation of tissue after four days in culture (Lambrot et al., 2006), and others showing central necrosis after only 48 h (Hallmark et al., 2007). Hanging drop cultures have been used to culture adult testis tissue for up to 14 days (Jorgensen et al., 2014), and has recently been extended to human foetal tissue (Jorgensen et al., 2015). Work in this chapter has used this technique to examine the effects of chemotherapy on the human testis.

5.1.2 Germ cells in the human foetal testis

During foetal life the seminiferous cords form, Sertoli cells proliferate, and germ cells undergo differentiation. In the first trimester the majority of germ cells are gonocytes, which express pluripotency markers such as OCT4 (Gaskell et al., 2004, Rajpert-De Meyts et al., 2004). Gonocytes then differentiate into prespermatogonia that express germ cell-specific markers such as MAGE-A4, VASA and DAZL (Gaskell et al., 2004, Anderson et al., 2007). In humans this is a gradual switch from gonocyte to spermatogonia from late first trimester to early postnatal life resulting in a heterogeneous population of germ cells (Mitchell et al., 2008), whereas in

rodents this occurs in a much narrow time window in all germ cells between e15.5 and e19.5 in the rat (Ferrara et al., 2006) and between e17 and birth in the mouse (Zayed et al., 2007). Some OCT4-positive gonocytes are even present in the infantile human testis (Rajpert-De Meyts et al., 2004). This heterogeneity means multiple markers must be used to visualise the germ cell population of the human testis.

5.2 Aims

The aims of this chapter were:

- To examine the culture of human foetal testis tissue.
- To start preliminary investigation into the effects of DOX or CIS on the human testis.

Only two samples were available for this project at different stages of development and therefore no quantification was attempted in this chapter.

5.3 Methods

5.3.1 Tissue collection

Human testis samples were obtained from two women undergoing elective termination of pregnancy. Terminations were not due to foetal abnormalities and gestation was determined by ultrasound scan and measurement of foot length. Ethical approval for obtaining tissue was given by South East Scotland Research ethics committee and NRES committee North East-Newcastle and North Tyneside with written and informed consent provided by the pregnant women. Samples were taken from fetuses at 11 weeks and 13 weeks + 2 days of gestation.

5.3.2 Genotyping SRY human foetal gonads

Genotyping for the SRY gene of human tissue was performed to confirm the sex of the gonads. This was performed prior to collection at the Centre for Reproductive Health, using previously documented protocols (Gaskell et al., 2004).

5.3.3 Preparation of human tissue for culture

Human testis samples were transferred on ice in transport medium, composed of Leibowitz L-15 solution supplemented with 10% foetal calf serum; 1% non-essential amino acids and 1% penicillin-streptomycin, and dissected into pieces of approximately 0.5 mm³. Some pieces were fixed for 90 min in 10% NBF as day 0 controls and others placed into culture.

5.3.4 Culture of human foetal testis

25 µl of the enriched medium (see 2.5.2) was placed on the lid of a Petri dish and each piece of tissue placed in the droplet. The lid was then inverted so the tissue sunk to the bottom of the drop.

5.3.4.1 First trimester testis

On the second day of culture, testis fragments were either transferred to new medium, or to new medium, that had been spiked with 0.05 µg ml⁻¹ of DOX. After 24 hours all pieces were transferred to new drops of fresh medium (Day 3). For the final day in culture (Day 4) medium was supplemented with 15 µg ml⁻¹ BrdU (Sigma Aldrich Ltd., UK).

5.3.4.2 Second trimester testis

On the second day of culture testis fragments were transferred to new medium or to medium spiked with CIS (0.5 µg ml⁻¹, high; 0.05 µg ml⁻¹, low) or DOX (0.1 µg ml⁻¹, high; 0.01 µg ml⁻¹, low). Lower doses than in the mouse study were chosen due to total depletion of germ cells in the mouse. After 24 hours all pieces were transferred to new drops of fresh medium (Day 3). Pieces were transferred to new drops every other day. Fragments were fixed on Day 4, Day 8 or Day 14 of culture. 15 µg ml⁻¹ BrdU was added to the culture for the final 24 hours for pieces fixed on Day 4 and 8 but this was not available for those fixed on Day 14 due to personal commitments and therefore has not been analysed here.

All tissue was washed in DPBS before being fixed as described (2.4). Between one and three fragments were cultured at each time point and concentration of CIS or DOX.

5.3.5 Haematoxylin and eosin

H&E staining was performed as described (2.5).

5.3.6 Immunohistochemistry

Immunohistochemistry was performed as described (2.6) using antibody combinations detailed in Table 5.1 except for OCT4 and MAGE-A4 double immunofluorescence which was performed over three days with only a single antibody added each day using protocol detailed below.

Following antigen retrieval in a pressure cooker with 0.01 M citrate buffer, an endogenous peroxidase block of 3% (v/v) H₂O₂ in methanol for 30 min was performed and sections were washed twice in tris buffered saline (TBS; 0.05 M Tris and 0.85% NaCl pH 7.4) for 5 min. Sections were incubated in blocking serum composed of 20% normal chicken serum diluted in TBS containing 5% (w/v) BSA for 30 min before incubation with MAGE-A4 at 1:200 overnight at 4 °C in a humidified chamber. Slides were washed twice in TBS, then sections were incubated with chicken anti-mouse peroxidase diluted at 1:200 for 30 min, followed by two TBS washes for 5 min and subsequently kept in the dark. Tyramide signal amplification kit (Perkin Elmer, UK) was used as the visualisation reagent at 1:50 for 10 min. Slides were washed in TBS for 5 min before being placed in citrate buffer and microwaved for 2.5 min. Sections were left to cool for 30 min and then incubated again in blocking serum, OCT4 at 1:200 (Abcam, UK), Chicken anti-goat Peroxidase at 1:200 and Tyramide as described previously. Slides were then counterstained with DAPI and washed in TBS before being mounted with PermaFluor mounting medium.

Table 5.1 Antibody combinations for immunohistochemistry.

Primary 1	Dilution	Secondary 1	Detection 1	Primary 2	Dilution	Secondary 2	Retrieval
BrdU	1:500	G α -Rat Bio	Strep 488nm	MVH	1:100	G α -M 568nm	Yes
SOX9	1:500	G α -R Bio	Strep 488nm	N/A	N/A	N/A	No
COUPTFII	1:200	G α -M Bio	Strep 568nm	CC3	1:500	G α -R 488nm	Yes
OCT4	1:200	D α -G 488nm	N/A	N/A	N/A	N/A	Yes
MVH	1:100	G α -M 568nm	N/A	N/A	N/A	N/A	Yes

D α -G 488nm = Donkey α -goat IgG1 488nm

5.3.7 Image acquisition

Images were taken as described (2.7).

5.4 Results

Two human foetal testis samples were examined. One was from a late first trimester testis (11 weeks gestation), which was forming seminiferous cords during the culture period. The second was from an early second trimester testis (13 weeks gestation) and had already formed cords.

5.4.1 First trimester testis

5.4.1.1 Culture of first trimester testis supports cord formation

Testis fragments from the first trimester testis (week 11 of pregnancy) were cultured for four days. The tissue initially had a disorganised cell distribution with only the occasional clearly marked cord to having many cords surrounded by a flattened layer of PTMs (Fig. 5.1). No obvious morphological differences were seen between the control cultured tissue and that treated with a low dose of DOX.

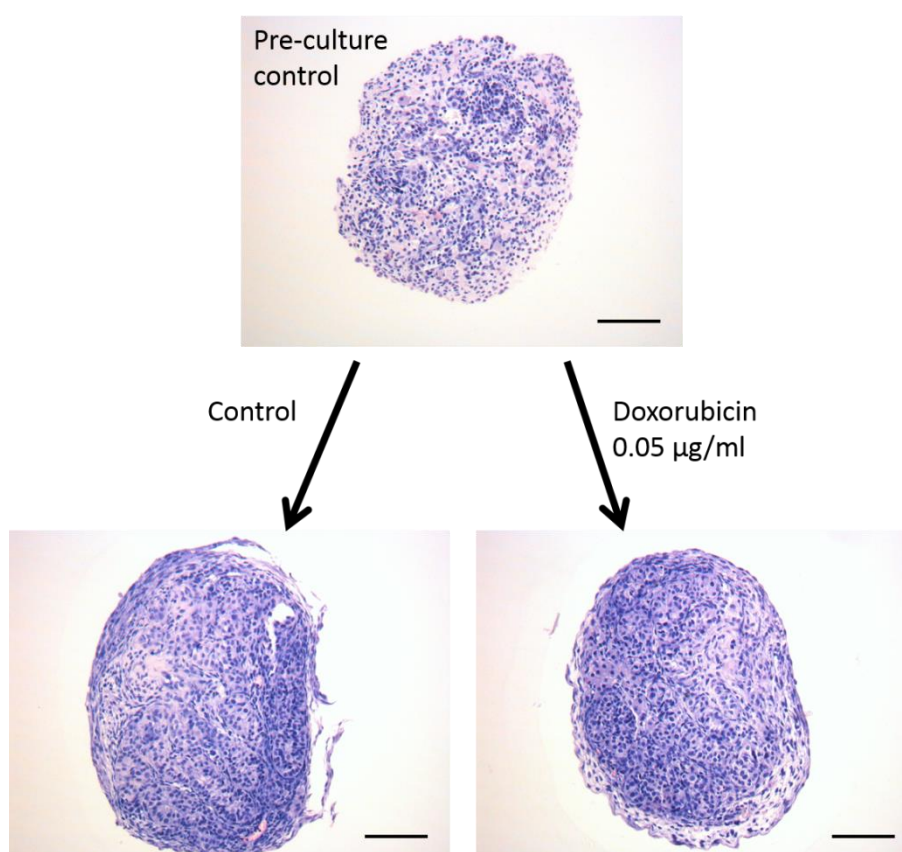


Figure 5.1 H&E culture of 11 week human foetal testis. Scale bars = 100 μm .

5.4.1.2 Evidence of germ cell on exposure to low dose DOX

To examine if low dose DOX caused depletion of the germ cell population, immunohistochemical analysis for MVH and BrdU was carried out in the same manner as for the mouse tissue reported earlier (see **Chapter 4**). There were very few cells clearly positive for MVH in any of the sections analysed, along with a lot of low level staining in the cords (Fig. 5.2). This made it impossible to make any firm conclusions. MVH would only mark the differentiated germ cell population and therefore is not giving a complete picture of the germ cell component of the tissue. Because of this, immunohistochemistry was carried out to examine the expression of OCT4, which marks the immature gonocytes and MAGE-A4, marking the more differentiated germ cells. There were very few germ cells in total when both OCT4 and MAGE-A4 were examined, but overall, expression did not appear to be affected by DOX (Fig. 5.3).

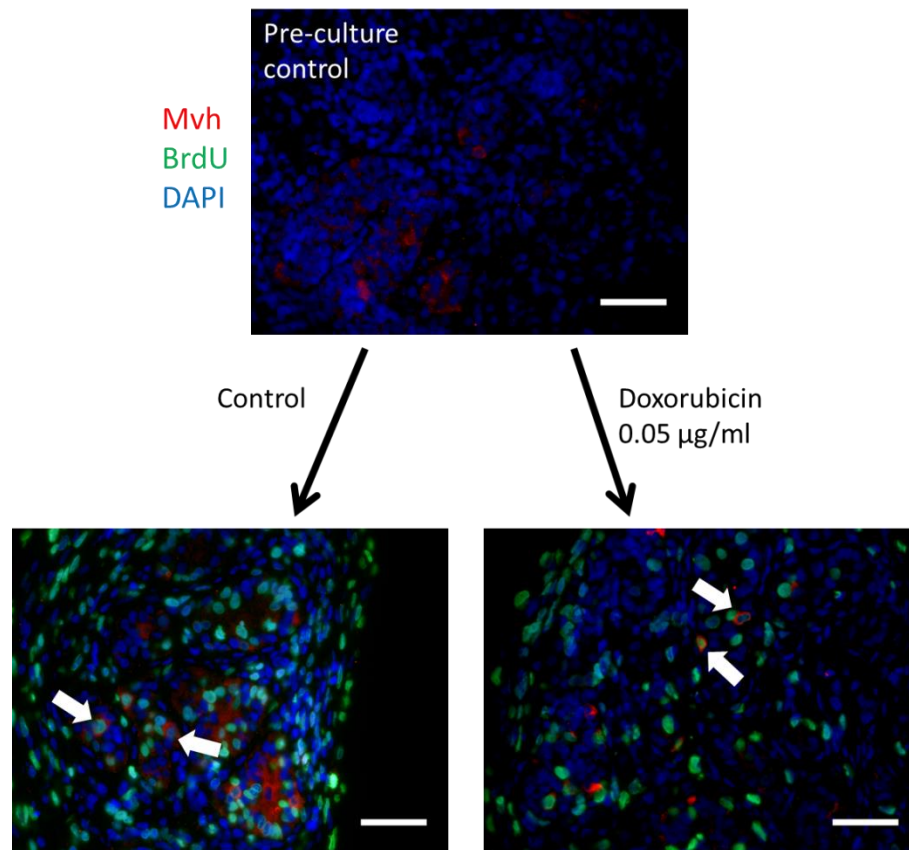


Figure 5.2 Immunohistochemical analysis for BrdU and MVH in first trimester testis culture. Occasional germ cells were positive for MVH (red) and were proliferating (BrdU; green). White arrows indicate MVH-positive germ cells. Pre-culture control was not given BrdU and therefore only MVH can be seen. Sections were counterstained with DAPI (blue). Scale bars = 50 µm.

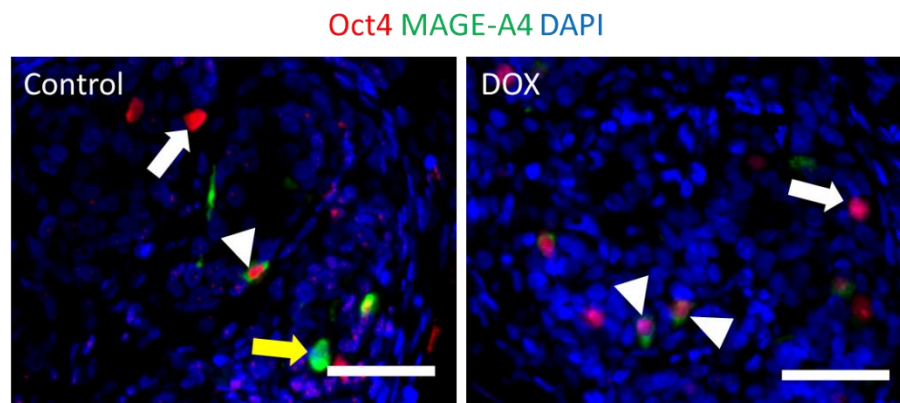


Figure 5.3 Expression of OCT4 and MAGE-A4 in first trimester testis culture. Only a few germ cells positive for OCT4 (red) or MAGE-A4 (green) could be seen in any of the fragments. White arrows mark OCT4-positive cells, yellow arrow marks MAGE-A4-positive cell and white arrowheads mark cells positive for both OCT4 and MAGE-A4. Sections were counterstained with DAPI (blue). Scale bars = 50 µm.

5.4.1.3 Somatic cells

The somatic cells in the testis were examined through expression of SOX9 in the Sertoli cells, but no clear differences in numbers were observed in any of the tissue, but appeared more organised in the cultured tissue due to the formation of cords (Fig. 5.4). COUPTFII, marking the interstitial cells appeared unaffected by culture in appearance or by exposure to a low dose of DOX and there was an apparent increase in CC3 expression following low dose DOX compared to control (Fig. 5.5).

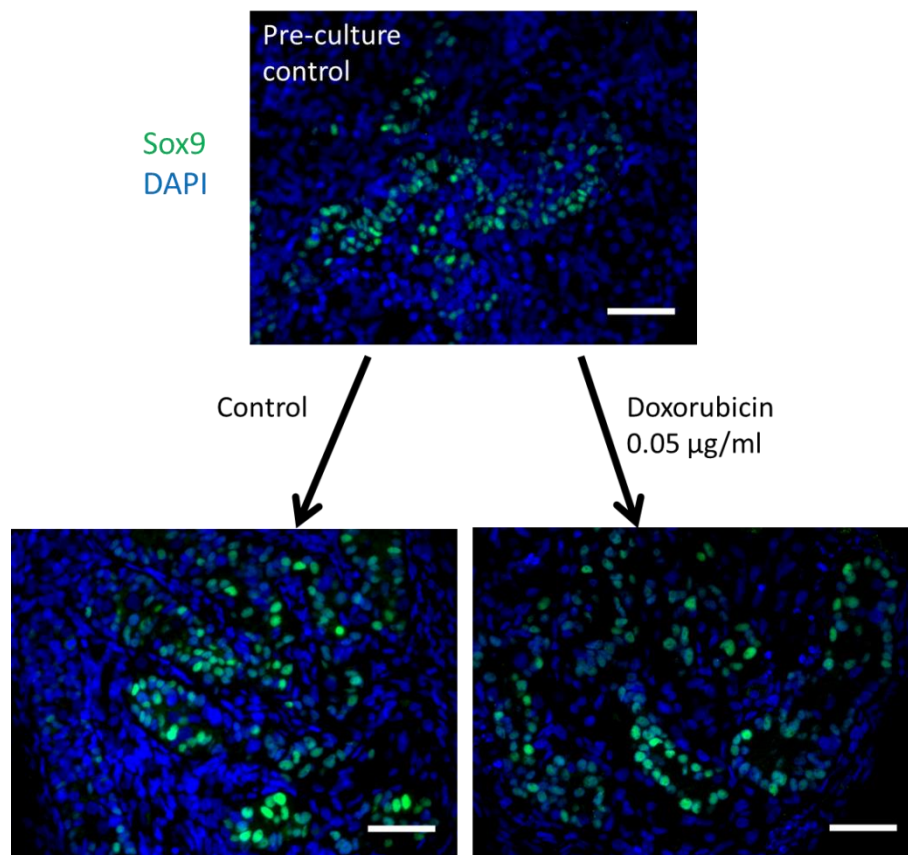


Figure 5.4 Expression of SOX9 in first trimester testis culture. SOX9 (green) was used to visualise Sertoli cells. No obvious depletion was observed in the treated tissue. Sections were counterstained with DAPI (blue). Scale bars = 50 µm.

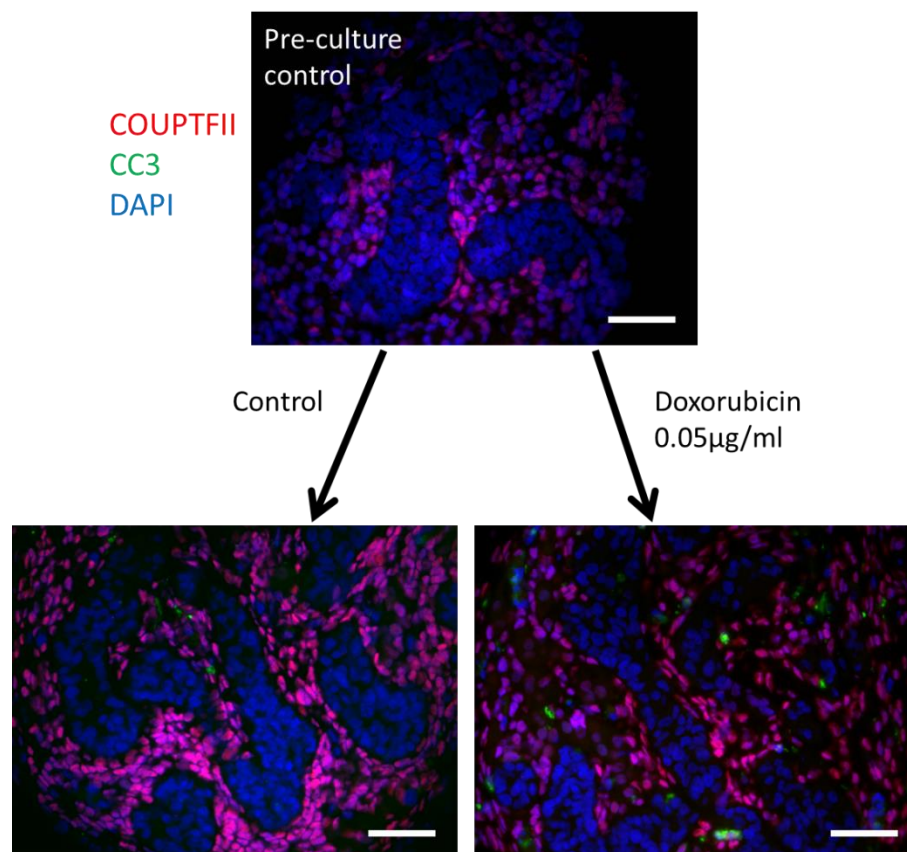


Figure 5.5 Expression of COUPTFII and CC3 in first trimester testis culture. COUPTFII expression (red) was expressed in the majority of interstitial cells in all sections examined. Occasional CC3-positive cells (green) were found in the cultured sections with an apparent increase in the treated tissue. Scale bars = 50 µm.

5.4.2 Second trimester testis

5.4.2.1 Second trimester human foetal testis tissue can be cultured for 14 days

The second trimester human testis was cultured for up to 14 days with pieces fixed at 4, 8 and 14 days after being placed in culture. Even after 14 days the tissue looked healthy with many preserved cords. The interstitial compartment appeared smaller by the end of culture with a much higher cell density (Fig. 5.6). As the tissue appeared to culture well for 14 days this time-point was used to examine the effects of CIS or DOX. At the low dose of CIS or DOX the tubules remained intact whereas, at the high dose of CIS or DOX there was a loss of cords and cell density (Fig. 5.7).

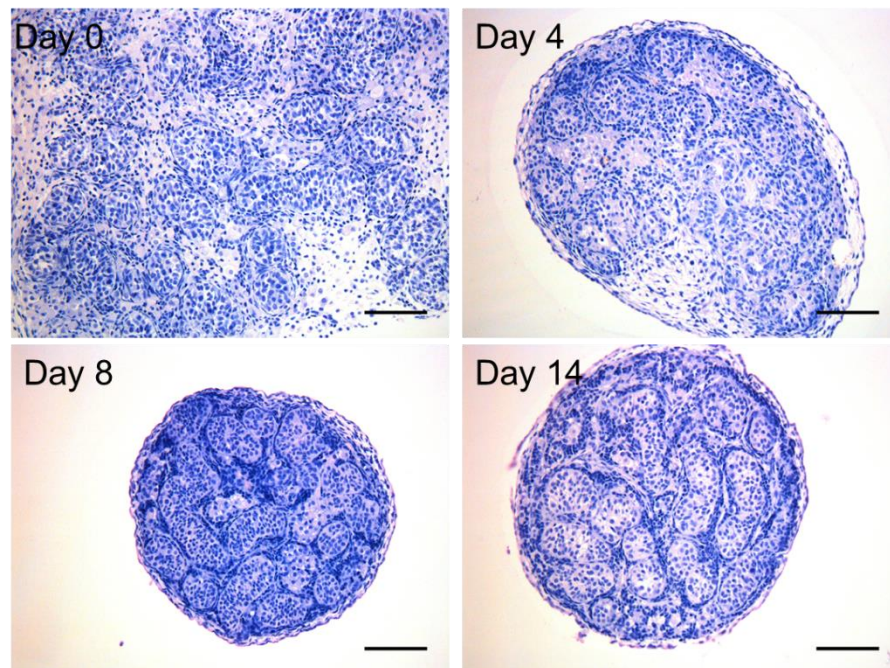


Figure 5.6 H&E of second trimester testis tissue after 14 days in culture. Tissue appears healthy. Scale bars = 100 µm.

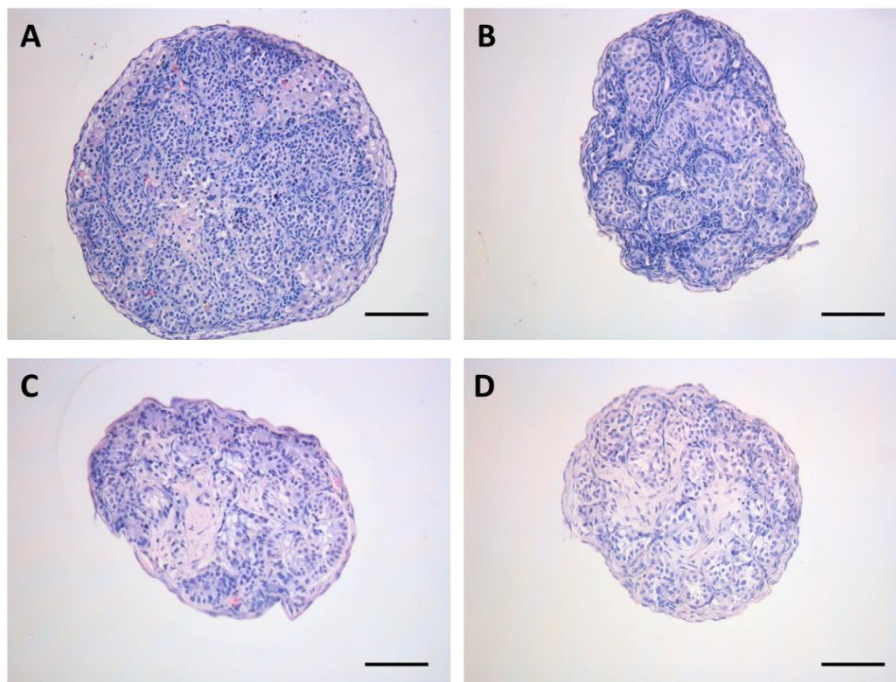


Figure 5.7 H&E of 14 day culture of second trimester testis in presence of CIS or DOX. The sections treated with A) low CIS or B) low DOX showed normal morphology. At the high dose of C) CIS and F) DOX there was a loss of cell density with fewer tubules and many pyknotic cells. Scale bars = 100 µm.

5.4.2.2 Germ cells

The germ cell population was examined by immunohistochemistry for MVH and OCT4. These antibodies would detect both immature gonocytes (OCT4) and the differentiating prespermatogonia (MVH). Before culture the tissue contained many OCT4-positive germ cells and weak MVH staining (Fig. 5.8A–B). Over 14 days of culture there were fewer OCT4-positive germ cells and MVH became much stronger suggesting either the tissue progressed through normal development or the OCT4-positive gonocytes were not supported by the culture (Fig. 5.8C–D). OCT4-positive germ cells were only present at the lowest dose of CIS, but in all other treatments there were none remaining (Fig. 5.9A, Fig. 5.9C, Fig. 5.9E, and Fig. 5.9G). MVH-positive germ cells were still present in the tissue treated with low dose CIS, but at the high dose of CIS and both the low and high dose of DOX there appeared to be a loss of these cells. However, there were always some MVH-positive germ cells (Fig. 5.9C–F). Overall, it would seem that the OCT4-positive immature germ cells may be more sensitive to CIS and DOX than the more mature MVH-expressing germ cells.

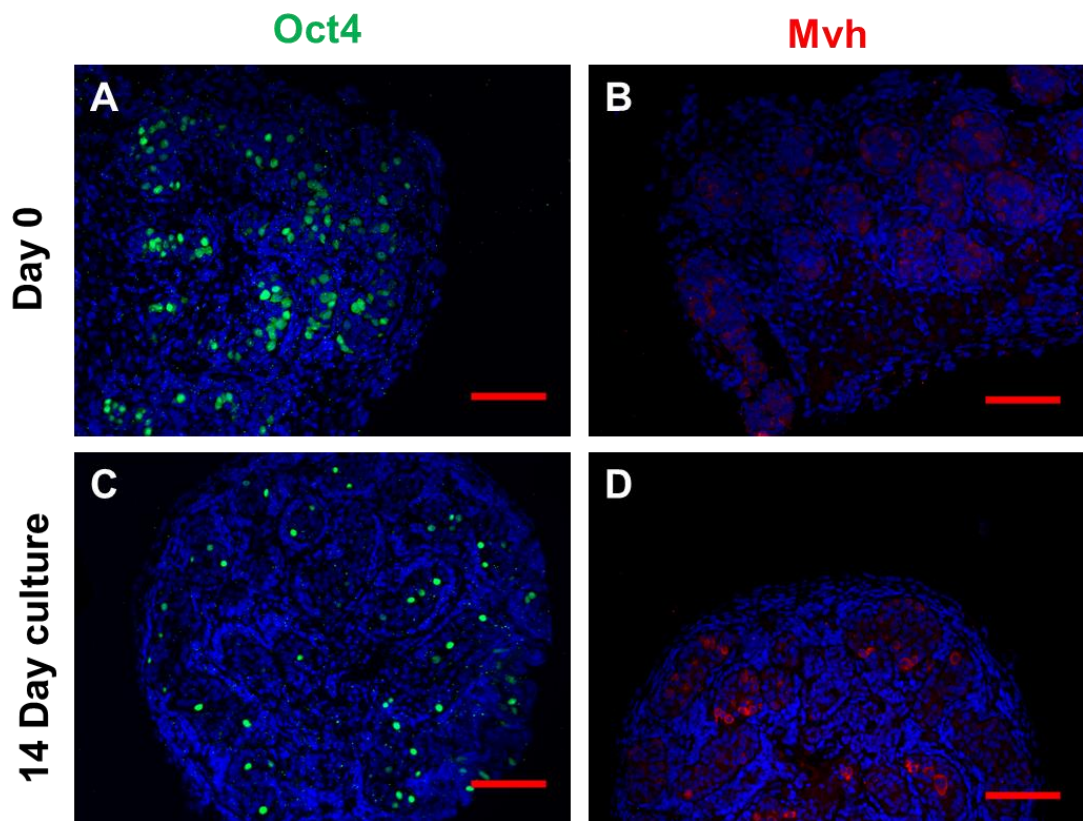


Figure 5.8 Germ cells in the second trimester testis cultured for 14 days. MVH (red) or OCT4 (green) expression counterstained with DAPI (blue) in A, B) pre-cultured testis fragment or C, D) 14 day control culture Scale bars = 100 μ m.

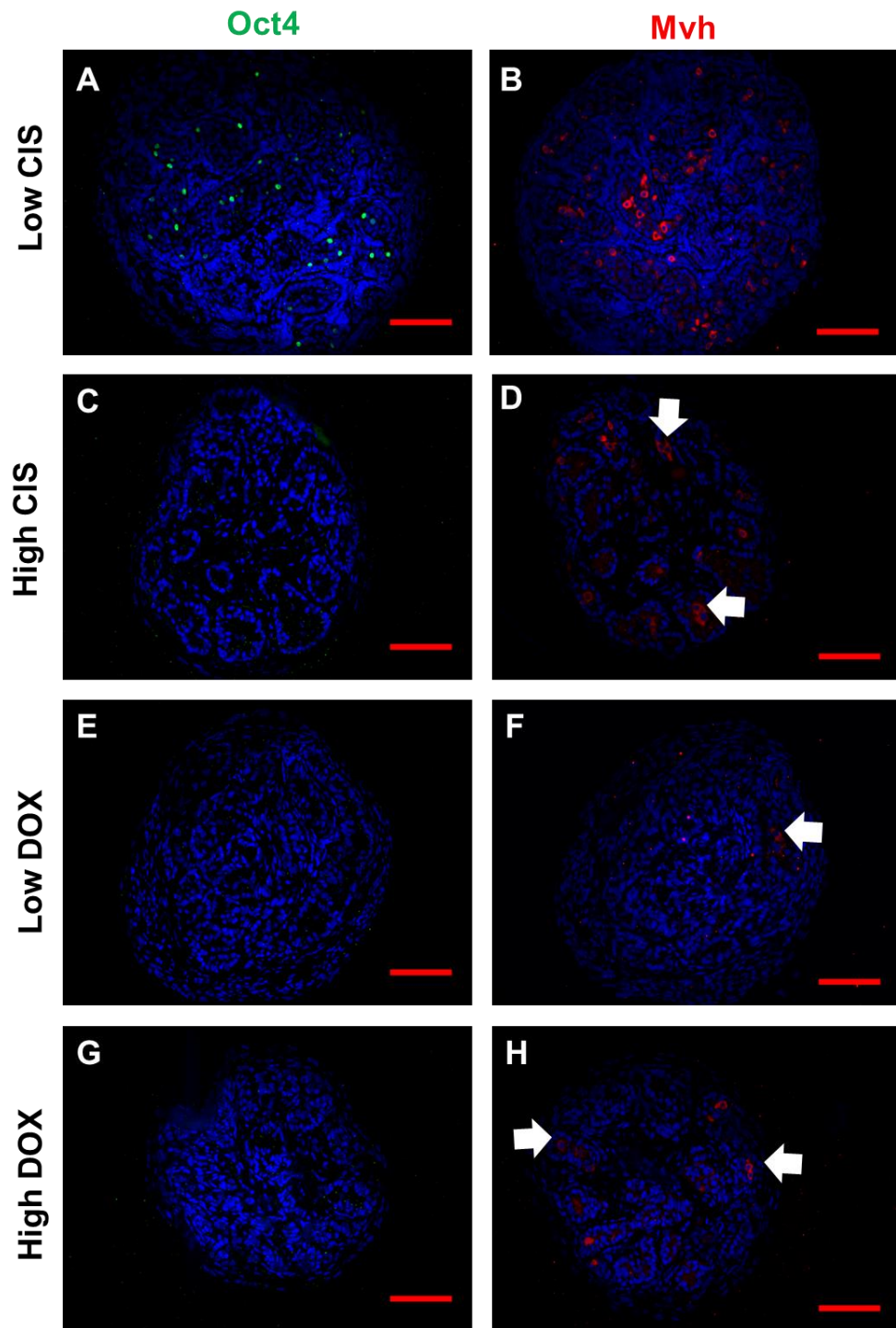


Figure 5.9 Germ cells in second trimester testis cultured for 14 days. OCT4 (green) or MVH (red) expression counterstained with DAPI (blue) in fragments exposed to A, B) low and C, D) high CIS or E, F) low and G, H) high DOX. White arrows indicate MVH-positive germ cells. Scale bar = 100 μ m.

5.4.2.3 Somatic cells

There was no apparent difference between the appearances of SOX9-positive Sertoli cells in any of the sections examined (Fig. 5.10). There was a change in SOX9 distribution in the tissue treated with high dose DOX, but this appeared to be due to disruption in cords rather than a change in expression. COUPTFII was still expressed in the majority of the interstitial cells following culture and in testis fragments exposed to the lowest dose of CIS (Fig. 5.11). However, there appeared to be fewer COUPTFII-positive cells following exposure to CIS (high dose) or DOX (low and high dose) indicating a loss of interstitial cells. There were very few CC3-positive cells in any of the sections examined.

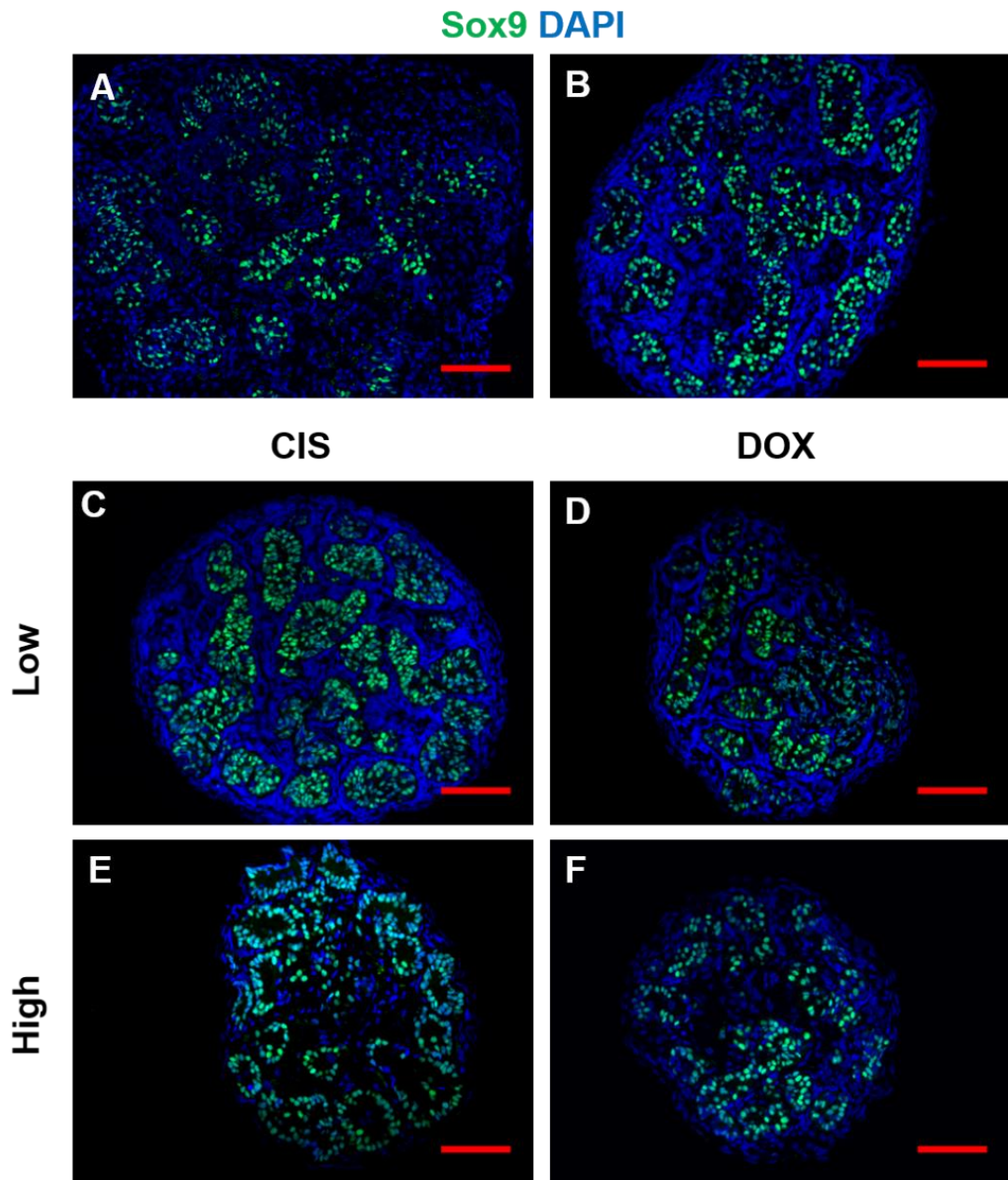


Figure 5.10 Immunohistochemistry for SOX9 in second trimester testis cultured for 14 days. SOX9 (green) expression counterstained with DAPI (blue) in A) pre-cultured testis fragment, B) 14 day control culture along with fragments exposed to C,E) CIS and D,F) DOX at a low and high dose respectively. Scale bars = 100 μm .

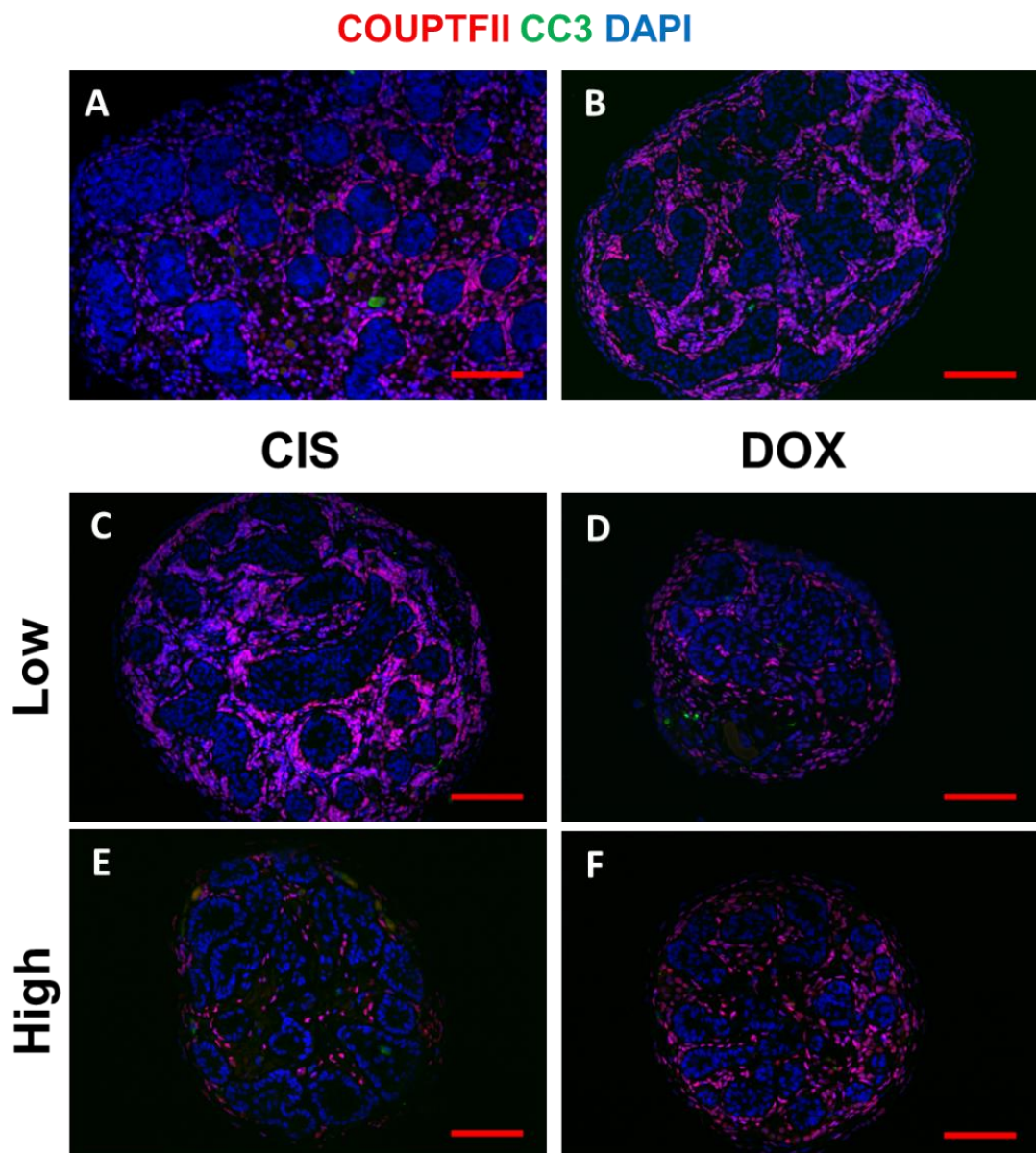


Figure 5.11 Immunohistochemistry for COUPTFII and CC3 in second trimester testis cultured for 14 days. COUPTFII (red) and CC3 (green) expression counterstained with DAPI (blue) in A) pre-cultured testis fragment and B) 14 day control culture. Fragments were exposed to C,E) CIS and D,F) DOX at a low and high dose respectively. Scale bars = 100 μ m.

5.5 Discussion

Two human foetal testis samples were used to provide preliminary data on the effect of chemotherapy on the human testis.

The first trimester sample formed seminiferous cords during the culture period and supported survival of all cell types. Due to the small size of the sample only one concentration of DOX, was examined and it appeared not to have an effect on the germ cells or somatic cells in the testis. This dose was the lowest dose examined in Chapter 4 that resulted in a depletion of the germ cell population. This sample is the least relevant to human patients as it is still forming tubules and the germ cells are immature. Combined with the small size, meaning limited number of fragments available, it is suggested to continue this work with testes taken later in gestation.

The second trimester culture of up to 14 days allowed examination of the medium term effects of chemotherapy on the human testis. The 14 days culture supported germ cell development with a reduction in OCT4-positive gonocytes and an increase in MVH-positive prespermatogonia. This is indicative of normal germ cell development in the second trimester human testes (Anderson et al., 2007, Mitchell et al., 2008). The culture also maintained the somatic cells in the testis and showed little apoptosis.

The germ cell population in the human foetal testis tissue was not completely depleted even at doses of CIS or DOX that had a dramatic effect on the mouse testis. As chemotherapeutics predominantly target dividing cells it is vital to know which cells are proliferating in the human foetal testis. In human foetal testis samples and foetal tissue grafted into immunodeficient mice there is a much higher proliferation index in the OCT4-positive germ cells than the more differentiated MAGE-A4 cells (Mitchell et al., 2010). This would fit with the observation of a total depletion of

OCT4-positive gonocytes, but preservation of the MVH-positive germ cells. However, the human testis is unlikely to be exposed to chemotherapy during pregnancy and treatment during infancy is relatively rare. This model was developed to infer the effect of chemotherapy in prepubertal patients treated for cancer. It is therefore crucial to know details of the germ cell population of the testis throughout childhood, and whether the germ cells are proliferating. In the mouse there is a big increase in proliferation in the neonatal period as the germ cells resume proliferation at approximately pnd5 (Jarvis et al., 2005), but there is little data about when differentiated pre-spermatogonia resume mitosis in the human testes. Analysis of postnatal testis samples suggests that germ cells are highly proliferative for the first year of life (Berensztein et al., 2002), which corresponds with the observation that there are a few remaining OCT4-positive cells (Rajpert-De Meyts et al., 2004), but the majority are negative by this age. Data from the marmoset suggest that although there is proliferation following birth (Kelnar et al., 2002), this is at a lower level than during the foetal period (Mitchell et al., 2008), implying the postnatal human testis may be even less susceptible than the foetal tissue presented here.

If this study was to be continued it would be better to image both germ cell populations in a single photomicrograph. This would allow total germ cells to be counted, while avoiding any double counting. The OCT4 and MAGE-A4 double immunofluorescence would be suitable for this. Ratios between OCT4-positive germ cells and MAGE-A4-positive cells tissue could also be calculated and compared to tissue fixed at different gestational ages to fully explore if the cultured tissue is following a normal developmental progression.

When the somatic cells were examined the supporting Sertoli cells appeared unaffected by either CIS or DOX, whereas, there was a loss of interstitial cells

expressing COUPTFII. These COUPTFII expressing cells include Leydig cells and the ALSCs (Kilcoyne et al., 2014) and therefore any loss of these cells could have a profound effect on testosterone production in later life. This was a surprising finding as it is believed that the Leydig cells are fairly resistant to chemotherapeutic damage in comparison to germ cells (Howell and Shalet, 2001). Nevertheless, there is evidence that there is increasing risk of hypogonadism in childhood cancer survivors, which can lead to depression, impaired libido and fatigue (Romerius et al., 2009). The interstitial population could be further examined by co-staining COUPTFII with 3β -HSD to distinguish the ALSCs (COUPTFII+/ 3β -HSD-) from the other interstitial populations to examine if a specific cell type was lost. Testosterone could be measured in the medium to see if this was affected by exposure to CIS or DOX. However, this would only be measuring the testosterone capacity of the immature Leydig cells and not the ALSCs that differentiate and proliferate during postnatal life to maintain androgen production throughout adulthood (Griswold and Behringer, 2009).

If more time were available it would be interesting to examine the tissue fixed on day four. This could be used to compare the longer term culture shown here to any initial damage present at day four, which would be similar to the mouse tissue presented earlier. This could determine if the germ cells surviving here represent recovery following a more substantial initial depletion.

In conclusion, these results appear to suggest that CIS or DOX affect primarily the undifferentiated germ cell population along with the interstitial cells. These cultures provide a starting point for further investigations into the effect of chemotherapy on the human foetal testis.

Chapter 6. General discussion

6.1 Summary of results

Initially the culture of postnatal mouse testis tissue was investigated to find the most suitable method for studying the effect of chemotherapy on the neonatal mouse testis. The results show that postnatal mouse testis tissue can be cultured for 4 days. Culturing was most successful using a membrane to allow the tissue to sit at the media–air interface and using a minimal medium. This culturing technique retained many of the aspects of neonatal development, although improvements could be made as there were fewer germ cells and Sertoli cells than would be found *in vivo*. The main focus of the project was to examine the effect of chemotherapy on the prepubertal testis. This was achieved using the developed *in vitro* culture of neonatal mouse testis. Tissue was exposed to three chemotherapeutic agents given to prepubertal patients: the CYP metabolite PM, CIS, and DOX. The tissue was exposed for 24 h and then examined two days later. All three chemotherapeutic agents showed a specific loss of germ cells indicating that fertility may be lost. However, at the doses examined, exposure to CIS and DOX even at lowest doses resulted in loss of the majority of germ cells whereas; PM only resulted in depletion at the highest dose. The loss of germ cells occurred without affecting Sertoli or interstitial cell numbers. There was little apoptosis by the end of the culture period, but considerably more when the tissue was fixed 24 h after the drug was added to the culture, indicating that the death of the germ cells happened earlier in the culture and that the dying cells were no longer present by the end of the culture period.

Finally, the culture of human foetal testis tissue was examined as far as was able, after obtaining only two samples: one from late first trimester testis and the other from the early second trimester. This cultured well in hanging drops for up to 14 days using recently developed methods (Jorgensen et al., 2015). When exposed to

DOX the first trimester testis did not appear affected, although a lower dose was used than in the mouse studies. The second trimester testis showed a complete loss of the immature germ cells on exposure to CIS and DOX whereas, there was only a reduction in the mature germ cells on exposure to DOX or at the highest dose of CIS. The interstitium also appeared to lose cells on exposure to DOX and the highest dose of CIS, which was not seen in the postnatal mouse testis. Using an *in vitro* model to assess chemotherapy induced damage has its strengths and weaknesses. It allows the isolation of effects directly on the testis without interference from other systems and reduces the number of animals used. However, as discussed in **chapter 4** the doses used were based on patient serum drug levels and therefore may not be relevant to the doses distributed to the testis. Additionally, in the testis, the seminiferous tubules do not have a direct blood supply, being separated by a basement membrane. In this study the testis was cut into small pieces exposing the seminiferous tubules more directly to the chemotherapeutic agents than would occur *in vivo* and therefore potentially resulting in a higher level of exposure.

In conclusion, the results presented here demonstrate the culture of postnatal mouse and embryonic human testis tissue and that the primary effect of CYP, CIS and DOX is through loss of the germ cells.

6.2 Further work

6.2.1 Investigation of long term damage

The model examined tissue approximately 72 hours after the addition of the chemotherapeutic agents. At this time point exposure to all concentrations of CIS or DOX and the highest concentrations of PM resulted in depletion of the majority of the germ cells from the testis. However, in human prepubertal patients fertility is only relevant months to years following exposure. The testis contains SSCs, which if still present following exposure, could contribute to regeneration of the seminiferous tubules. A few dividing germ cells were present at the end of culture,

which indicates there could be potential for regeneration. It has been shown that even a very small number of stem cells can contribute to the regeneration of spermatogenesis (Brinster, 2002). This long term damage could be difficult to assess in an *in vitro* culture system as it is hard to maintain good tissue integrity over a long period of time. However, recently, testis tissue culture has been achieved for up to 60 days (Sato et al., 2011). An alternative model would be grafting the testis tissue following a short culture into immuno-deficient mice. Grafts from neonatal mice have been known to survive and complete spermatogenesis (Honaramooz et al., 2002). After a number of weeks the grafts could be retrieved and examined to see if the loss of germ cells has persisted, recovered or if there was further loss of the remaining cells. This could also be applied to any other model species, as xenografts have been established for a wide range of species, including human testis, recapitulating many aspects of development (Goossens et al., 2008, Mitchell et al., 2010). Therefore, this method would be applicable for tissue that may be more relevant to human patients see below.

6.2.2 Mechanism of damage

By the time the tissue was examined, the germ cells had already died in samples exposed to PM, CIS or DOX, and there was little evidence of apoptosis in the tissue. Therefore, in order to elucidate the mechanism, it would be appropriate to examine the tissue earlier to observe the process of germ cell death. A time course analysis could be performed for this purpose. It could be achieved by fixing the tissue at a variety of points after the addition of the drug to examine when the germ cells are dying and the pathway by which this occurs. One initial culture was fixed at 24 hours after the addition of the drug and showed that at the highest dose of DOX the majority of germ cells were already lost, whereas, at the highest dose of CIS there were still many remaining. This was the case despite the two drugs having a similar degree of damage two days later. This suggests that each drug may take a different length of time to enter the cell and cause the initial damage; or after the drug has

initiated the damage it may occur through different apoptotic pathways. A range of molecules within the apoptotic pathway could be examined at these time points to build up a more complete picture of the damage to the testis following exposure to PM, CIS or DOX. This information is vital for the informed development of preventative treatments.

6.2.3 Human tissue

The majority of work conducted here was using a neonatal mouse model. However, postnatal development is different for rodents and humans. The rodent does not have the long extended childhood, but progresses straight from the neonatal period into puberty, both in terms of germ cell development and Sertoli cell maturation. This means the time point that is more important for childhood cancer survivors is not well represented in the mouse. The ideal model for patients would be human tissue, and one such potential model was examined here using foetal testis tissue. Although there is not much tissue available, each culture uses only a small amount of tissue as every piece is cut into small fragments. Each culture can therefore use the same sample for both treated and control tissue. However, there are differences between the foetal and neonatal testis. There is little information about the prepubertal testis in the human. The majority of data is inferred from primate models such as the marmoset (Kelnar et al., 2002, Chemes, 2001). This model indicates that there is proliferation in the neonatal testis, but that this is lower than during foetal life (Mitchell et al., 2008). To be able to find the most appropriate model for human patients the normal development of the prepubertal testis will need to be more intensively studied. Samples are beginning to be obtained from prepubertal patients for experimental fertility preservation methods. If some of these samples could be used for research, then more information could be gained about this developmental period. It would not only inform the best models to use in studies on the damage of chemotherapy, but also aid in the development of fertility preservation techniques needed to ensure the tissue will be useful in later life.

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