Male fertility in survivors of childhood cancer

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Thesis submitted to the Faculty of Medicine, University of Edinburgh, for the degree of Doctor of Medicine (M.D).

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November 2003

Acknowledgements

During this project I have benefited greatly from the expertise and generous advice of many friends and colleagues, and along the way, incurred my share of debts and sacrifices.

A special debt of gratitude is owed to Dr Hamish Wallace, for his excellent supervision, endless warm support and encouragement, and words of wisdom during this project. I also wish to give special thanks to Dr Christopher Kelnar, Dr D. Stewart Irvine, Dr Richard Anderson and Dr Richard Sharpe for their support and guidance during the supervision of my work. I am deeply grateful for their invaluable suggestions during the course of this study.

Special thanks and appreciation to the team in the Clinical Andrology Unit: Susan Hamilton, Janet McGhee, Mairi Johnstone and Anna Rattray, who ensured the smooth running of the clinical aspects of my project, and to Maggie Paterson, Diana Harkess, Alastair Campbell, Victoria Hunter, Sarah Dickson, Fiona Watt, Karen Kerr, Valerie Nicholson and Peter Bishton who offered expert assistance with the more technical aspects of the laboratory work.

I am grateful to the MRC, Human Reproductive Sciences Unit, Edinburgh, for the opportunity to study in their laboratories.

My gratitude is extended to the team at the MRC, in particular to Chris McKinnell for his guidance and patience during the immunocytochemistry studies and to Dr Jane Fisher for her 'expert technical support' and good humour. I also want to say a big thanks to the histology team, in particular to Sheila MacPherson and Mike Millar, for their advice and assistance during the preparation of the sections. Enormous thank you also to Nancy Evans for her much appreciated assistance with the immunohistochemistry studies. Many thanks to Dr Kathryn Mackenzie for her

assistance with the pathology specimens. A special thank you also to Ted Pinner for his assistance with the illustrations.

The opportunity to expand my knowledge and experience would not have been possible without the financial support of the Child Growth Foundation.

I would like to thank the patients, who obligingly and enthusiastically participated in the clinical studies. Without them the work would not have been possible.

I am eternally grateful to the unquestioning, unconditional support and encouragement from my mum and stepdad. My thanks also extend to my family and friends, who provided constant moral support and understanding. Their tolerance and acceptance of my commitment to my work ensured the completion of my thesis.

Declaration

I certify that this thesis does not contain material previously published or written by any other person, except where referenced in the text, and that the results of this thesis have not been submitted for any other degree or diploma. The results of this study have been published in journals during the course of this work and are indicated at the start of the thesis and in the text.

To my mum and Ian, with love and thanks.

Angela B. Thomson

Chapter **Page Number**

Chapter **Page Number**

Abstract

The successful treatment of childhood cancer with chemotherapy and radiotherapy may be associated with testicular damage resulting in impaired spermatogenesis and temporary or permanent infertility in adulthood. In this study testicular function and semen quality was investigated in 33 survivors of childhood cancer. Treatment of childhood cancer was associated with a significant risk of impaired spermatogenesis, with 30.3% of this population being azoospermic and 18.2% being oligozoospermic. Moreover, in those men who do have surviving spermatogenesis after treatment, it is commonly compromised, with reductions being observed in ejaculate volume, sperm concentration, sperm motility and the proportion of morphologically normal sperm. Only 33.3% of this group of 33 male childhood cancer survivors had completely normal semen quality by conventional criteria. However, the sperm produced do not appear to carry a greater burden of damaged DNA compared with the healthy population, suggesting that assisted conception treatment is a safe option for these men.

Detection of gonadal damage in the prepubertal male is hampered by lack of a sensitive marker. The role of inhibin B as a marker of early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer was investigated. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B during and immediately after treatment stopped, although one boy showed a delayed deleterious effect. Inhibin B changed earlier and appeared to be a more sensitive marker of gonadal damage than FSH or LH. Prospective studies are

underway combining inhibin B with FSH, LH and sex hormone measurements, to assess the impact of cancer therapy on gonadal function in children, particularly as they approach and progress through puberty.

For prepubertal boys fertility preservation through semen cryopreservation is not an option and consequently, attention is focusing on the development of techniques that might preserve or restore fertility potential in boys being subjected to gonadotoxic cancer therapy. In rats, it has been shown that some germ cells survive cytotoxic therapy and that the resulting azoospermia is a consequence of the inability of those spermatogonia that are present to proliferate and differentiate. Suppression of the hypothalamic-pituitary-gonadal (H-P-G) axis facilitates recovery of spermatogenesis following such cytotoxic treatment. Investigation of whether suppression of the H-P-G axis in men rendered azoospermic by treatment for childhood cancer might restore spermatogenesis was undertaken, using both semen analysis and testicular biopsy as end points. In men treated with sterilising radiotherapy and chemotherapy for childhood cancer, effective gonadotrophin suppression with medroxyprogesterone acetate for at least 3 months did not result in restoration of spermatogenesis. The absence of histological evidence of spermatogonial stem cells in testicular biopsies from these men before and after suppression suggests complete ablation of the germinal epithelium and irreversible infertility.

Understanding the vulnerability of the prepubertal human testis to cytotoxic damage is compounded by the dearth of data describing normal testicular development in the

prepubertal human. Based on immunohistochemical studies in marmosets, a primate that exhibits a similar developmental profile to the human male, it has been shown that significant testicular development occurs during childhood long before the clinical onset of puberty. If we can establish that cell activity does occur in the 'quiescent' testis in boys and is comparable to changes shown in the marmoset, it will validate use of the marmoset as a model for the human in this instance and give encouragement to the possibility of using this primate model to develop a method of protecting spermatogenesis in boys undergoing cancer therapy prior to puberty. Preliminary studies to investigate the development of the prepubertal human testis confirmed testicular cell activity in the foetal and neonatal periods and infancy comparable to that shown in the marmoset. However, to date development during mid childhood and early puberty has proved to be somewhat discordant with the marmoset studies. It is too premature to definitively conclude that marmoset and human testicular development are dissimilar, as a number of explanations have been proffered to explain the discrepancies, including suboptimal tissue fixation and antigen preservation in the human tissues.

Publications arising from this thesis

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Glossary

ABVD	adriamycin + bleomycin + vinblastine + dacarbazine
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ChlVPP	chlorambucil, vinblastine, procarbazine and prednisolone
COP	cyclophosphamide, vincristine and procarbazine
COPP	cyclophosphamide, vincristine, procarbazine and prednisolone
DLCO	carbon monoxide diffusion capacity
ECG	electrocardiogram
EFS	event free survival
EPIC	etoposide, prednisolone, ifosfamide and cisplatin
Gy	Gray (unit of radiation)
HD	Hodgkin's disease
ICSI	intracytoplasmic sperm injection
IQR	interquartile range
IVF	*in vitro* fertilisation
MACOP-B	mechlorethamine, doxorubicin, prednisolone, vincristine and cyclophosphamide
MDS	myelodysplastic syndromes
MLL	mixed lineage leukaemia
MOPP	mechlorethamine, vincristine, procarbazine and prednisolone
OEPA	vincristine, etoposide, prednisolone and adriamycin
OPPA	vincristine, procarbazine, prednisolone and adriamycin

OS	overall survival
SR	survival rate
VACOP-B	vinblastine, doxorubicin, prednisolone, vincristine, cyclophosphamide and bleomycin
VAPEC-B	vincristine, doxorubicin, prednisolone, etoposide, cyclophosphamide and bleomycin
VBVP	vinblastine, bleomycin, etoposide and prednisolone
VEEP	vincristine, etoposide, epirubicin and prednisolone

List of figures

Figure **Page Number**

List of tables

Chapter 1

Introduction

1. Introduction

Tremendous advances in the management of childhood malignancies over the last 30 years mean that the majority of children can realistically hope for long-term survival (Figure 1.1)[1-2]. With survival rates in excess of 70% it is estimated that by the year 2010 one in 250 of the young adult population will be a long-term survivor of childhood cancer[3]. However, the successful treatment of childhood cancer with multi-agent chemotherapy, in combination with surgery and or radiotherapy, is associated with significant morbidity in later life[4]. The major challenge faced by paediatric oncologists today is to sustain the excellent survival rates whilst striving to improve the quality of life of the survivors.

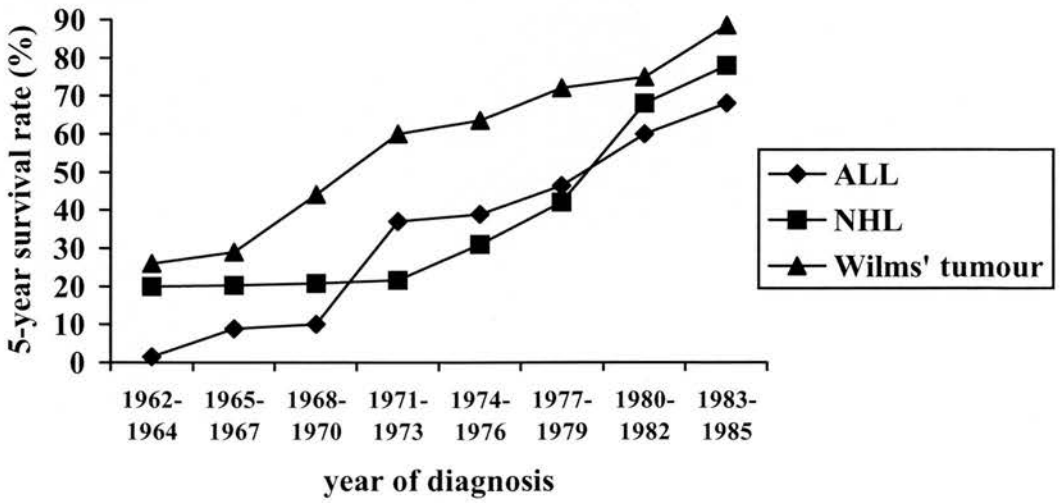


Figure 1.1. Trends in 5 year survival rates[1]

Testicular dysfunction is well recognized after chemotherapy and radiotherapy treatment for childhood cancer. Cytotoxic agents may cause irreparable damage to the testes with both infertility and hypogonadism reported[5-16]. In contrast to the testicular germinal epithelium, Leydig cells are more resistant to the deleterious effects of chemotherapy and radiotherapy. However, direct irradiation of the testes may cause hypogonadism and testosterone replacement may be required to initiate and sustain pubertal development and normal potency in adulthood[8-18]. After chemotherapy, while overt testosterone deficiency is uncommon, mild Leydig cell dysfunction is increasingly recognised and it is not yet clear whether these patients may benefit from testosterone replacement therapy[17-19].

While Leydig cell dysfunction is amenable to treatment, damage to the germinal epithelium is usually irreversible and associated with long lasting or permanent azoospermia[10,11,15,16]. Infertility is a recognised consequence of treatment for childhood cancer. Advances in assisted reproduction techniques have focused attention on preserving gonadal tissue for future use[20-23]. For prepubertal boys, for whom fertility preservation through cryopreservation of semen is not possible, testicular germ cell harvesting and cryopreservation may in the future preserve fertile potential[24-27]. Harvesting, and the potential future use of gonadal tissue, is an exciting area of gamete biology that opens up new and uncharted territory for paediatric oncologists. However, many scientific, ethical and legal issues remain to be addressed before techniques to preserve fertility are available[28].

An overview of normal testicular physiology and clinical assessment of testicular function is presented in Chapter 2. Chapter 3 reviews the impact of childhood cancer treatment on testicular function, explores the options for preserving fertile potential and discusses the effects such interventions may have on the offspring. Chapter 4 is devoted to assessment of testicular function in long-term survivors of childhood cancer. This involved detailed study of sperm quality and spermatozoal DNA integrity and we discuss the implications for these men when considering assisted conception.

Although the extent of testicular damage and probability of infertility may be predicted to some extent by the treatment the child has received, confirmation of this is delayed until testicular function can be assessed in adulthood. At present, there are no clinical tools available to assess testicular function in prepubertal children. In Chapter 5, the results of a pilot study evaluating the role of inhibin B as a potential marker of gonadotoxicity in children during treatment for childhood cancer are presented.

There is tremendous impetus to develop techniques to preserve or restore fertility in cancer patients as discussed in Chapter 6. For the group of men in our study rendered infertile as a consequence of treatment for cancer during childhood, it was investigated whether hormone manipulation might restore spermatogenesis in azoospermic men and present our findings in Chapter 6. There is very little known about the extent of the damage to the testes at histological level. The impact of

cancer therapy on testicular tissue was assessed using immunohistochemical techniques and the findings are also presented in Chapter 6.

There are no options currently available in clinical practice for preservation of fertility in the prepubertal child. To establish why the testis in prepubertal boys is susceptible to the damaging effects of certain cancer therapies, such that fertility is compromised in adulthood we carried out detailed assessment of human testicular tissue from prepubertal boys, presented in Chapter 7. Our objective was to prove that the testis in boys is active before the onset of puberty, as opposed to being quiescent, as was traditionally thought to be the case. With this knowledge, a strategy to 'quieten' the prepubertal testis, based on hormone manipulation could be devised and this may be able to protect the testis from damage and thus help preserve fertility in adulthood.

During this period of research it became abundantly clear that preservation of fertility in children undergoing treatment for cancer raises many ethical and legal issues that are the subject of much debate. Chapter 8 of this thesis addresses these issues and discusses the implications for the medical profession, the child and his/her family. Based upon the evidence available to date we propose a strategy for the best clinical practice for preserving fertile potential in children undergoing treatment for cancer.

Chapter 2

Overview of normal testicular function

2. Overview of normal testicular function

The major functions of the male reproductive tract are twofold: to manufacture spermatozoa and deliver these to the female reproductive tract, and to produce male sex steroid hormones, necessary for normal male sexual differentiation during foetal life, puberty, adult male phenotype and behaviour, and reproduction[29,30]. The organs responsible for these functions are (i) the testes (Figure 2.1); site of sperm and androgen production: (ii) the duct system or transport system: and (iii) the accessory glands; for secretion of seminal fluid necessary to support transport and facilitate fertilization. Testicular function is regulated by the anterior pituitary hormones; follicle stimulating hormone (FSH) and luteinizing hormone (LH), under the controlling influence of hypothalamic gonadotrophin releasing hormone (GnRH). In turn, the hypothalamic-pituitary-testicular axis is influenced by testicular hormones, which completes the negative feedback loop.

2.1. Anatomy of the testis

The testes are composed of two structurally distinct but functionally related compartments, the seminiferous tubules and intertubular space, site of spermatogenesis and steroidogenesis respectively (Figure 2.2). The intertubular compartment is subdivided into two compartments: the interstitial and intravascular space. The seminiferous tubule, of which there are about 500 in each testis, is a convoluted loop that converges and drains spermatozoa into the rete testis. The tubules are lined by seminiferous epithelium consisting of various types of male

germ cells (spermatogenic cells) and a single type of supporting cell, the Sertoli cell. The seminiferous tubules are further subdivided into two compartments, the basal and adluminal compartments, by formation of inter-Sertoli cell tight junctions, which form the blood-testis barrier and segregate the epithelium into anatomical and physiological compartments (Figure 2.2 insert). The two compartments support different stages of spermatogenesis[29,30].

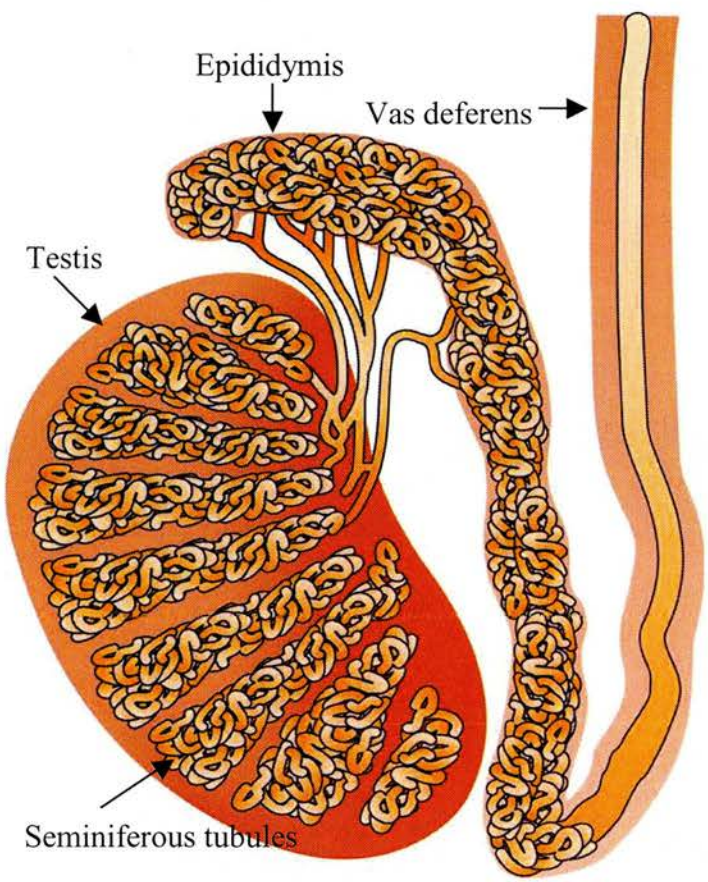


Figure 2.1. Anatomy of the human testis

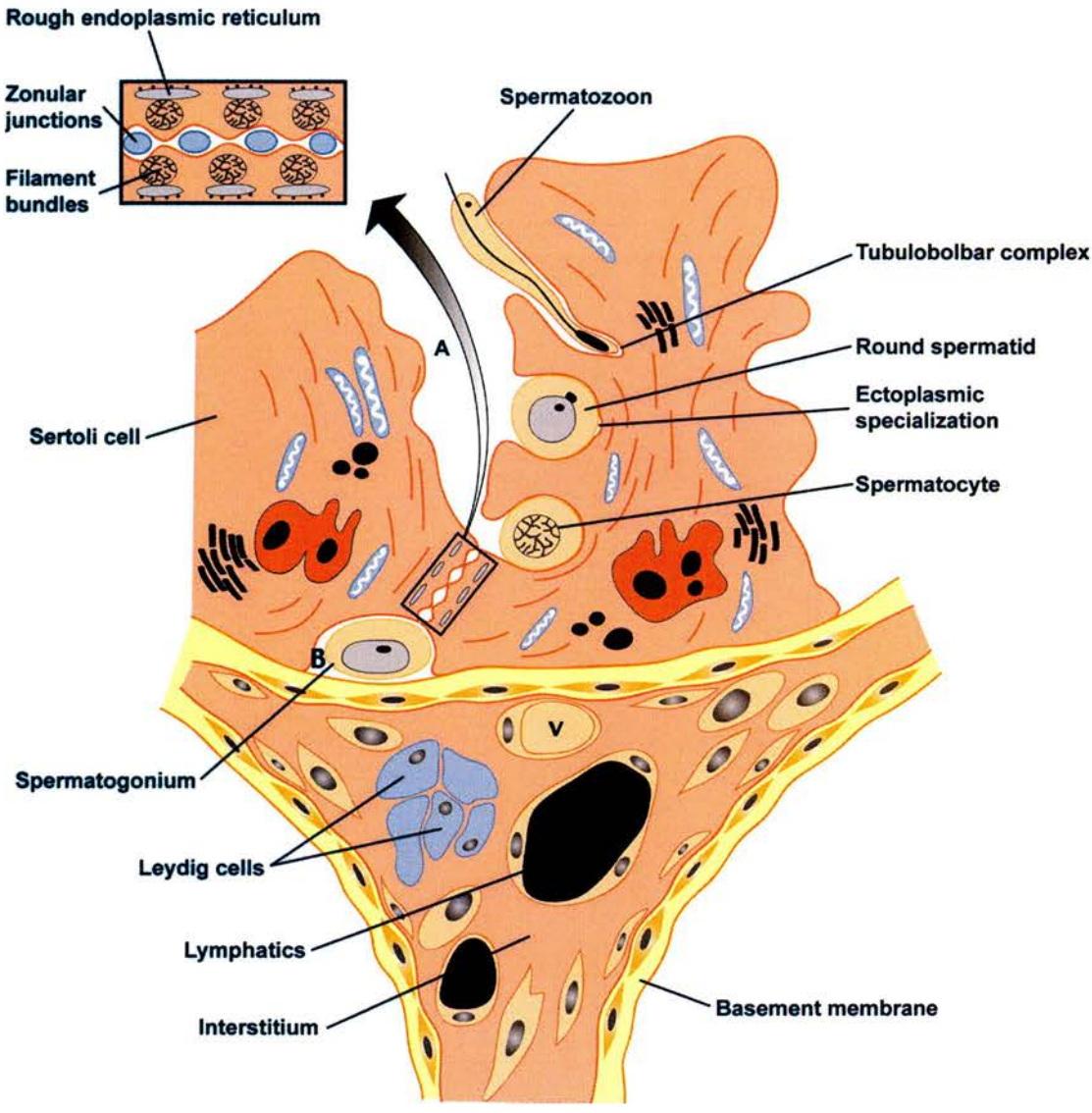


Figure 2.2. Cross-section through part of a seminiferous tubule of an adult testis.
The testis is divided into two major compartments; the seminiferous tubule and intertubular space. The intertubular space is subdivided into the interstitium, which includes the lymphatic vessels and Leydig cells, and the vascular compartment (V). The seminiferous tubule is further divided into basal (B) and adluminal (A) compartments. The blood vessels, lymphatics and nerves are contained entirely within the intertubular space, separated from the seminiferous tubules by the basement membrane. Within the tubule, the basal and adluminal compartments are separated by rows of zonular tight and gap junction complexes (see insert) which link adjacent Sertoli cells round the entire circumference of the tubule. The spermatogonia are confined to the basal compartment, whilst spermatocytes, round and elongating spermatids and spermatozoa are in the adluminal compartment, in intimate contact with the Sertoli cells. (Support for illustrations provided by Ted Pinner, MRC Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh).

2.2. Spermatogenesis

Spermatogenesis is a complex process by which diploid germ cell spermatogonia undergo proliferation and differentiation into mature haploid spermatozoa[29]. The general organization of spermatogenesis is essentially the same in all mammals and can be divided into phases of development through which all spermatogenic germ cells pass sequentially over time. This highly co-ordinated process can be divided into three phases: mitotic proliferation of spermatogonia to yield primary spermatocytes; meiotic maturation of spermatocytes to yield round spermatids; and differentiation of spermatids into mature spermatozoa, a process known as spermiogenesis (Figure 2.3). The time taken from division of one stem cell spermatogonium to production of mature spermatozoa varies between species, and in humans is determined to be approximately 74 days. Clearly, male reproduction does not hinge upon an episodic pattern of fertility every six weeks or so, but rather, is dependent upon continuous production of mature spermatozoa. Consequently, sperm production is organised spatially and temporally such that rounds of spermatogenesis are initiated at time intervals that are constant and characteristic for each species. There are six stages in humans. To ensure a continuous supply of spermatozoa, each successive segment of the tubule will show a sequential stage of development. It is as if adjacent tubule segments, each containing synchronized populations of spermatogenic stem cells, have entered the cycle slightly out of phase with each other, giving the tubule a helical appearance longitudinally (Figure 2.4). The resulting appearance is known as the spermatogenic wave which is a description of a spatial event, rather than the temporal cycle events[29,30].

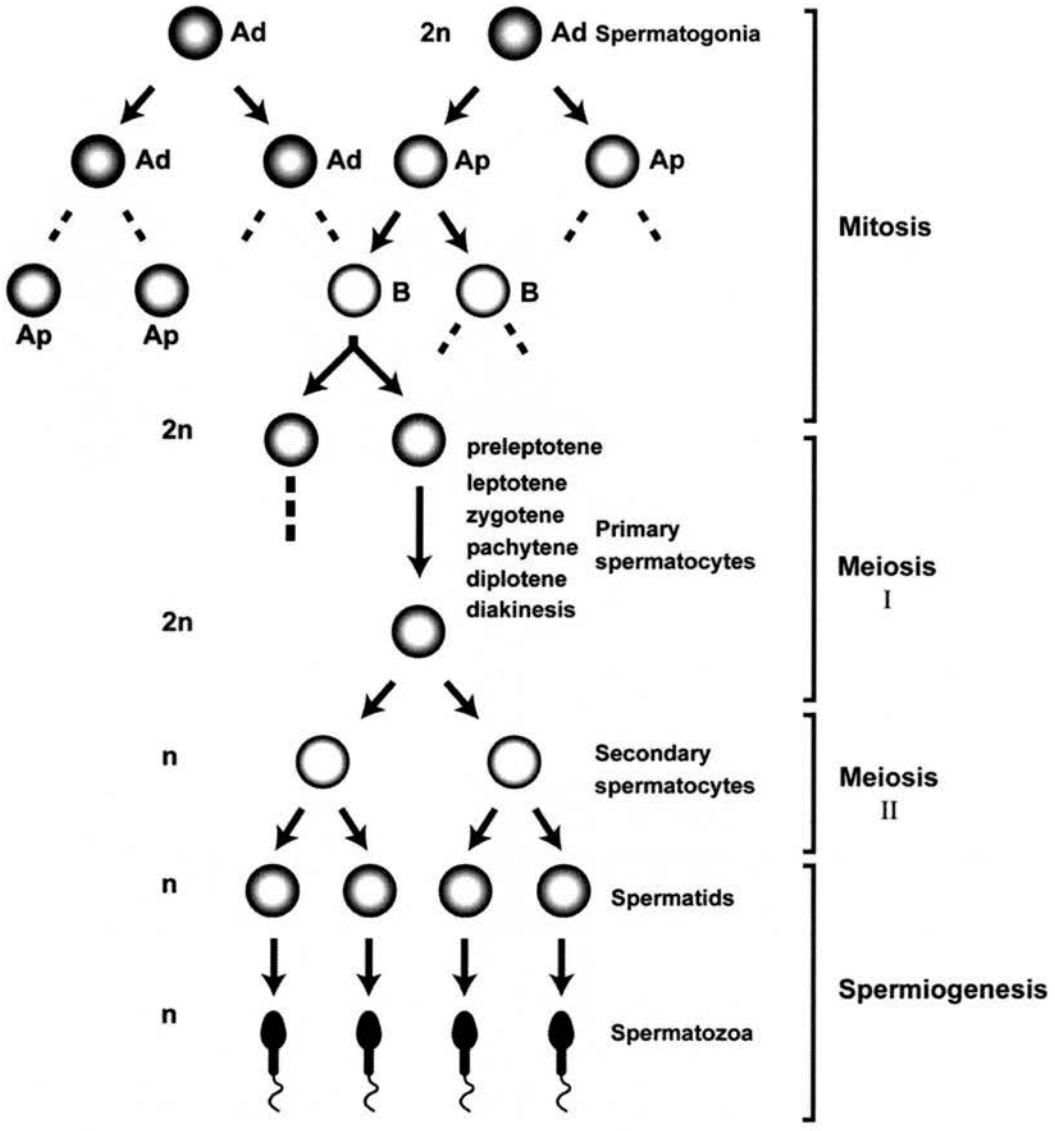


Figure 2.3. Division of spermatogonial germ cells to mature spermatozoa
This process can is divided into three phases: mitotic proliferation of spermatogonia to yield primary spermatocytes; meiotic maturation of spermatocytes to yield round spermatids; and differentiation of spermatids into mature spermatozoa, known as spermiogenesis. (Support for illustrations provided by Ted Pinner, MRC Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh).

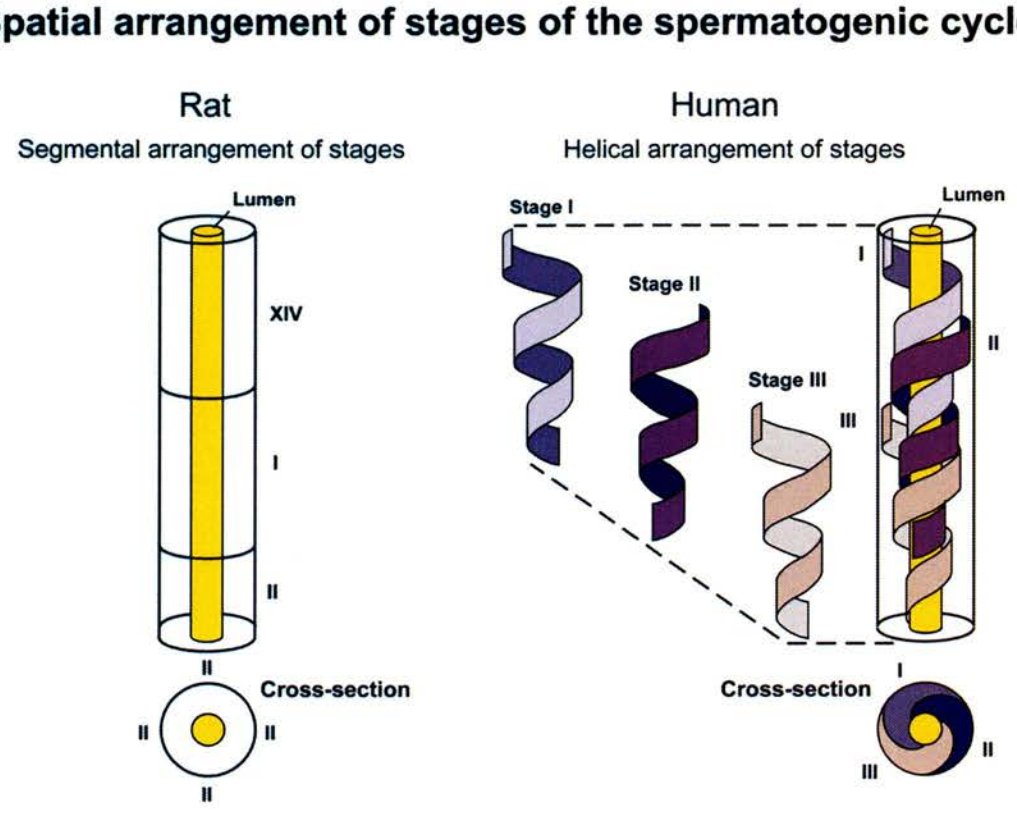


Figure 2.5. Helical appearance of tubule.

Diagrammatic representation of the different spatial arrangement of the stages of spermatogenic cycle along a short length of seminiferous tubule in the rat and human[29].

Organization of this highly complex process of spermatogenesis, both spatially and temporally, is incompletely understood but there is compelling evidence to suggest that it is attributable to the Sertoli cell, which provides a continuous cytoplasmic

network around the tubule that may enable communication and synchronization to occur. The Sertoli cell also spans across the tubule from membrane to lumen facilitating communication between intratubular space, basal and adluminal compartments. The functions of the Sertoli cell are numerous and include fluid production, phagocytosis of degenerating germ cells, synthesis and secretion of numerous proteins and enzymes, metabolic conversions, and production of known and putative growth factors. The principal role of the Sertoli cell is to support spermatogenesis in response to hormone regulation by FSH and testosterone[29,30].

2.3. Hormone regulation

Testicular function, and ultimately spermatogenesis, is regulated by the anterior pituitary hormones LH and FSH, which are released from the anterior pituitary gland in response to hypothalamic gonadotrophin-releasing hormone (GnRH) and modified by various regulatory factors from the testes namely testosterone and inhibin. FSH binds exclusively to receptors on the Sertoli cell and stimulates synthesis of androgen receptors, inhibin and activin, which play a mediatory role in spermatogenesis. Testosterone, essential for spermatogenesis, is synthesized and secreted by the Leydig cells in response to stimulation by LH, and passes through the cellular barriers to the Sertoli cell, with a substantial proportion entering the blood and lymphatic system. FSH and testosterone act synergistically on Sertoli cells to promote Sertoli cell function and support spermatogenesis because germ cells per se do not possess receptors for either hormone. The mechanism by which spermatogenesis is initiated and sustained is poorly understood and is likely to

involve a number of stimulatory and inhibitory mechanisms. Testosterone exerts a negative feedback regulation on the hypothalamic and pituitary hormones. Another such inhibitory mechanism is the regulatory role of inhibin B. Inhibins are glycoprotein heterodimers composed of an alpha subunit and one of two types of beta subunits, either βA (inhibin A) or βB (inhibin B). In males, inhibin B is the major component and the Sertoli cell is the predominant site of its production, although there is some speculation regarding subunit production by Leydig and germ cells. Inhibin B, the secretion of which requires the presence of germ cells, mediates non-steroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion[31,32]. Inhibin B secretion in the adult requires the presence of intact germ cells[31] and increasingly inhibin B is being used as a predictor of seminiferous epithelial function.

In addition to its mandatory role in spermatogenesis, testosterone has a peripheral role. Testosterone circulates in the plasma as a pro-hormone, bound largely to plasma proteins (98%), and is converted to the metabolically active hormones, DHT and oestradiol. DHT is thought to mediate male sexual differentiation and virilization. Oestradiol appears to mediate bone maturation and mineralization, epiphyseal fusion, and negative feedback inhibition of FSH[29,30].

2.4. Puberty

The prepubertal testis has classically been defined as a quiescent organ, however evidence is emerging to the contrary[33-35]. The development of the testis from the time of foetal differentiation to adulthood is characterised by dramatic morphological and functional changes that lay the groundwork for the onset of spermatogenesis. From birth to puberty the testis has been shown to triple its volume, largely attributable to an increase in seminiferous tubule length[35]. Sertoli cells proliferate intensely and are functionally active during childhood, producing large amounts of anti-Mullerian hormone, throughout the prepubertal period, and inhibin B until the age of 2-4 years[36]. These hormones are believed to modulate the proliferation and differentiation of Leydig cell precursors. Multiplication of early germ cells does occur but progression beyond the mitotic stage is not supported by the immature Sertoli cells.

At puberty there is a sleep-entrained increase in the pulsatile secretion of LH and to a lesser extent FSH, associated with increased nocturnal plasma testosterone concentrations. With pubertal progression, the increased pulsatile release of gonadotrophins is maintained throughout the day and night and high concentrations of testosterone are sustained throughout the 24-hour cycle. Spermatogenesis is initiated at puberty as a consequence of increased secretion of FSH. Integral to this is the changing role, and responsiveness of the Sertoli cell. FSH stimulated maturation of the Sertoli cells to form two compartments and create the blood-testis barrier is essential for the progression of germ cells through meiosis and spermatogenesis. As

puberty progresses the responsiveness of the Sertoli cell, and ultimately control of spermatogenesis, shifts from FSH to testosterone[34,35,37].

2.5. Investigation of testicular function

Assessment of testicular maturation and function involves pubertal staging, measurement of plasma hormones and semen analysis (Table 2.1). Pubertal staging provides important clinical information about both Leydig cell function and spermatogenesis[38]. The development of normal secondary sexual characteristics would imply intact Leydig cell function with normal steroidogenesis. Reduced testicular volume (<15ml), determined using the Prader Orchidometer (Figure 2.6), is strongly suggestive of impaired spermatogenesis and azoospermia. Hormone analysis requires measurement of basal plasma FSH, LH, testosterone and inhibin B if available[39]. In prepubertal children hormone analysis is an unreliable predictor of gonadal damage because the hypothalamic-pituitary-testicular axis is relatively quiescent. In post pubertal boys elevated LH and diminished testosterone levels would indicate Leydig cell dysfunction. Subtle Leydig cell damage may be manifest by elevated LH with normal serum testosterone levels. Elevated FSH and diminished inhibin B would be indicative of impaired spermatogenesis but semen analysis remains the definitive measure of spermatogenesis.

Table 2.1. Assessment of testicular function

Testicular dysfunction	
Leydig cell dysfunction	Reduced testosterone Elevated LH
Seminiferous epithelial dysfunction	Reduced testicular volume Elevated FSH Low inhibin B Impaired spermatogenesis

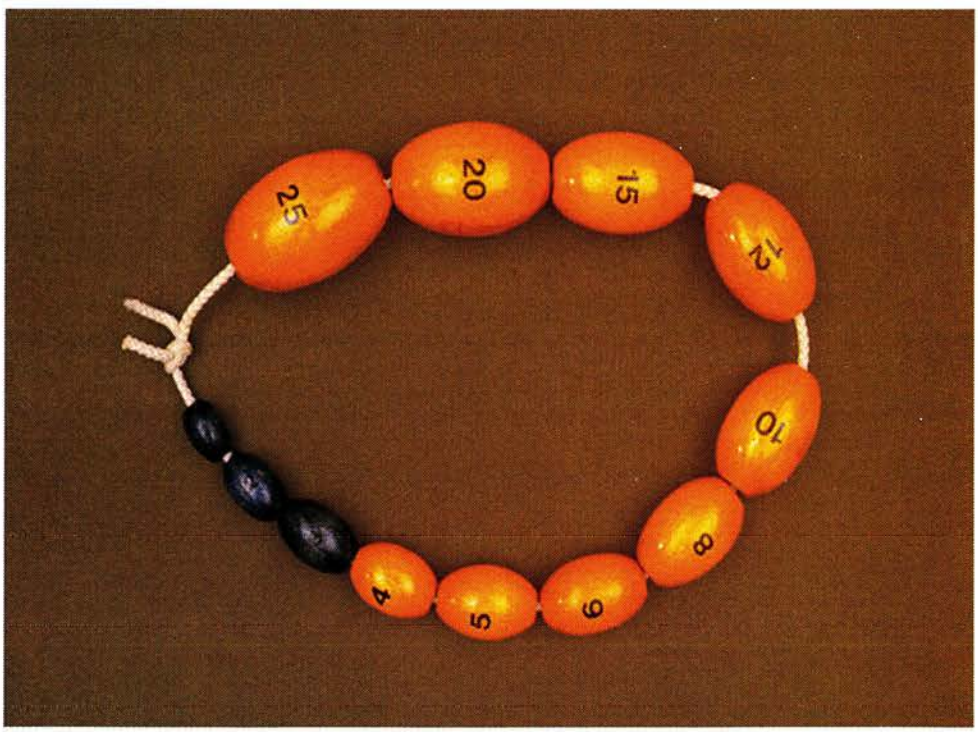


Figure 2.6. Prader Orchidometer.

This is used to clinically determine testicular volumes. Prepubertal testes, less than 4ml in volume are coloured blue on the beads.

Chapter 3

Testicular damage

3. Testicular damage

Cytotoxic chemotherapy and radiotherapy may damage the testes at all ages and result in hormone insufficiency and sterility. However, the full impact of gonadotoxic treatment in prepubertal children is difficult to detect during childhood and may be manifest during puberty as hypogonadism or in adulthood as infertility[5-16].

There is convincing evidence that the prepubertal testis is susceptible to the toxic effects of radiation and chemotherapy[8-11,16]. The seminiferous epithelium is very susceptible to cytotoxic damage but the exact mechanism of cell damage remains to be elucidated[40]. This is explored further in Chapter 7.

The mechanism of Leydig cell damage is equally uncertain. Chemotherapy may have a direct cytotoxic effect upon the Leydig cells or an indirect impact by damaging other cell populations and disrupting paracrine regulation. Radiotherapy may directly destroy the slowly turning over Leydig cells at high doses or more likely, have an indirect effect due to damage to the vasculature. Radiotherapy damage to the testes is associated with decreased testicular blood flow[41]. Where this is marked, compensatory mechanisms may be insufficient to increase intratesticular testosterone concentrations, resulting in reduced testosterone output. Furthermore reduction in arterial blood flow into the testes may be associated with a diminished stimulatory effect of LH[42].

3.1. Chemotherapy

3.1.1. Seminiferous epithelial damage

Chemotherapy-induced damage to the testicular seminiferous epithelium has been recognized for many years and was first described in humans by Spitz in 1948[43]. Post-mortem examination of testicular tissue from 30 men treated with nitrogen mustard demonstrated complete absence of spermatogenesis and seminiferous tubules lined with Sertoli cells only, in 90% of cases. Since then a number of agents have been identified as causing testicular damage, including cis-platinum, procarbazine and the alkylating agents, such as chlorambucil and cyclophosphamide (Table 3.1)[10,11,44-48]. Cytotoxic chemotherapy agents may produce long-lasting or permanent damage to the seminiferous epithelium resulting in oligozoospermia or azoospermia[10,11,49-53].

The extent of the damage to the testis is dependent upon the agent administered and dose received[10,11,44,47-54]. However, as most treatments are delivered as multi-agent regimens, often with synergistic toxicity, it can be difficult to determine the specific contribution of each individual agent. The impact of chemotherapy on testicular function has been widely investigated and most clinical studies have focused on combination chemotherapy regimens used in the treatment of haematological malignancies, particularly Hodgkin's disease[10,11,48-52]. Occasionally chemotherapeutic agents are administered as monotherapy, enabling the direct gonadotoxic effects of the agent to be studied, such as cyclophosphamide treatment of immunologically mediated disease[47].

Table 3.1. Gonadotoxic chemotherapy agents

Gonadotoxic chemotherapy
Alkylating agents
Cyclophosphamide
Ifosfamide
Nitrosoureas eg BCNU, CCNU
Chlorambucil
Melphalan
Busulphan
Antimetabolites
Cytarabine
Platinum agents
Cis-platinum
Others
Procarbazine

Combination chemotherapy treatment of Hodgkin's disease with established regimens (mechlorethamine, vinblastine, procarbazine and prednisolone (MVPP) or mechlorethamine, vincristine, procarbazine and prednisolone (MOPP) or chlorambucil, vinblastine, procarbazine and prednisolone (ChlVPP) or cyclophosphamide, vincristine, procarbazine and prednisolone (COPP)) have been reported in a number of studies to result in permanent azoospermia in more than 85% of adult males. The gonadotoxic agents in these regimens are mechlorethamine and procarbazine in MVPP and MOPP respectively, chlorambucil and procarbazine in

ChlVPP, and procarbazine and cyclophosphamide in COPP[10,11,48-51,55]. The ABVD combination (adriamycin, bleomycin, vinblastine and dacarbazine), which contains neither an alkylating agent nor procarbazine, has been shown to be significantly less gonadotoxic, resulting in temporary azoospermia in 33% of patients and oligozoospermia in 21%, with 'full' recovery after 18 months reported in all patients retested[50]. However, the advantage of reduced incidence of azoospermia is offset by the increased potential of cardiac disease due to anthracycline exposure.

In view of the high chance of azoospermia associated with treatment for Hodgkin's disease, alternating combination chemotherapy regimens, 'hybrid' regimens, have been developed in an attempt to reduce the overall dosage of any one particular agent and potentially reduce all drug related side effects. 'Hybrid' regimens (such as alternate cycles of ABVD with cycles of ChlVPP or MOPP) appear to be as effective as single regimen therapies with respect to cure rates and will hopefully be less gonadotoxic and cardiotoxic. Fertility is preserved in approximately 50% of men following three cycles of MOPP, in contrast to almost universal azoospermia following six cycles of MOPP[51]. In direct comparison of MOPP treatment with MOPP/ABVD, azoospermia was reported in 100% and 76% of patients, respectively[56].

A number of other combination chemotherapy regimens have also been studied which do not include procarbazine or chlorambucil and have generally been shown to be less gonadotoxic. Treatment of 51 men with non-Hogkin's lymphoma (NHL)

with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone)- based chemotherapy rendered all men azoospermic during treatment but recovery to normospermia occurred in 67% of patients and oligozoospermia in 5% by 5 years[57]. A number of other regimens have been used successfully to treat NHL and are also associated with a more favourable outcome in terms of testicular function, including MACOP-B or VACOP-B (mechlorethamine doxorubicin, prednisolone, vincristine, cyclophosphamide and bleomycin or vinblastine replacing mechlorethamine in the latter)[58] VAPEC-B (vincristine, doxorubicin, prednisolone, etoposide, cyclophosphamide and bleomycin)[59], and VEEP (vincristine, etoposide, epirubicin and prednisolone)[52]. The vast majority of men have normal fertility following treatment with the above regimens, which, although containing cyclophosphamide, emphasises the role of procarbazine in causing irreversible damage to the testicular seminiferous epithelium.

Bone marrow transplant (BMT) or peripheral blood stem cell (PBSC) rescue following marrow ablation are increasingly being used to successfully treat a number of haematological and solid malignancies. Testicular dysfunction may follow marrow ablation therapy with total body irradiation or high-dose chemotherapy, including cyclophosphamide and busulphan. In a study of 155 patients, aged 13-56 years, undergoing BMT for haematological malignancies or aplastic leukaemia, preparative chemotherapy regimens included cyclophosphamide (200mg/kg) in 109 patients, or busulphan (16mg/kg) and cyclophosphamide (200mg/kg) in the remaining 46 patients. Recovery of testicular function, defined by normal FSH

and/or sperm production, was assessed at 3 (1-19) years post treatment. In the group treated with cyclophosphamide only, 42 of the 109 (39%) patients had evidence of recovery, while amongst those who received both agents testicular dysfunction was evident in 38 of 46 (83%)[16]. Busulphan was clearly associated with greater gonadotoxicity at those doses. In another study semen analysis in long term survivors of bone marrow transplantation receiving preconditioning with busulphan (16mg/kg) and cyclophosphamide (120mg/kg) demonstrated sperm production in 21 out of 26 (81%) patients, 10 of whom were oligozoospermic[60].

Follow up of 30 men for a mean of 12.8 years following treatment with cyclophosphamide (total dose 560-840 mg/kg) for childhood nephrotic syndrome, reported azoospermia in 13%, oligozoospermia in 30% and normal semen analysis in the remaining 57% of the men[47]. The threshold for impaired spermatogenesis was a total dose of 10g. However, when used for the treatment of solid tumours in combination with doxorubicin and dacarbazine or vincristine, which have not been shown to be gonadotoxic, azoospermia was permanent in 90% of men treated with cyclophosphamide doses >7.5g/m^2 [61]. Management of childhood leukaemia, which is the commonest childhood malignancy, is continually evolving and often includes cyclophosphamide or cytarabine. A study of testicular histology in 44 boys treated for ALL demonstrated a 50% reduction in tubular fertility index (TFI) (percentage of tubules containing identifiable spermatozoa), with severe impairment (TFI <40%) in 18 patients. The severity of the damage was influenced by previous chemotherapy treatment with cyclophosphamide and cytarabine (>1g/m^2), whereas the tubular

fertility index improved with increasing time from treatment[62]. 'Full' recovery of spermatogenesis was observed in 3 of 7 of the patients with severe depression of TFI followed up for median (range), 10.8 (5.5-15.9) years off treatment[45]. Seminiferous epithelial damage has also been described following successful treatment for childhood ALL with a modified LSA_2L_2 protocol, which includes treatment with cyclophosphamide and cytarabine[63]. In contrast, normal testicular function was reported in 14 boys successfully treated for ALL, which did not include either cyclophosphamide or cytarabine[64]. Current treatment of ALL in the UK includes cytarabine (total dose: $2g/m^2$ or $4g/m^2$) and cyclophosphamide (total dose: $1.2g/m^2$ or $2.4g/m^2$). Although this is unlikely to be sterilising, long-term follow-up is necessary.

The value of serum inhibin B in detecting male gonadal dysfunction was studied in 27 postpubertal and 12 pubertal survivors of childhood cancer. Serum inhibin B levels were found to correlate with testicular volume and gonadotrophin concentrations. In the post pubertal group, small testicular volume was strongly correlated with low inhibin B levels (<42pg/ml) and inversely related to FSH levels. In all prepubertal survivors, inhibin B levels were greater than 90pg/ml, except in one patient with testicular cancer, whose inhibin B was barely detectable and FSH was elevated, indicative of testicular damage. Inhibin B appears to be a sensitive marker of seminiferous epithelial function and may be a tool for evaluation of testicular function in cancer survivors unable to produce semen for analysis[65].

3.1.2. Leydig cell damage

The Leydig cells are less vulnerable to the cytotoxic effects of chemotherapy than the sensitive seminiferous epithelium[11,44,47-49,54,55]. Gonadotoxic chemotherapy agents responsible for seminiferous epithelial damage may, however, also cause Leydig cell dysfunction. In a study of 30 men treated with cyclophosphamide (2-3mg/kg body weight/day for a mean of 280 days, range 42-556) for childhood nephrotic syndrome all men had normal development of secondary sexual characteristics, normal libido and sexual function. All patients demonstrated a significantly raised luteinizing hormone response on stimulation with luteinizing hormone releasing hormone, in the presence of normal testosterone concentrations, suggesting compensated Leydig cell failure[47].

Following treatment for Hodgkin's disease with the gonadotoxic regimens MVPP, MOPP, ChlVPP, COPP, elevated LH (basal and stimulated) was reported in 24-88% of patients[10,11,46,48,49,55]. In the majority (>85% of patients) testosterone levels were within the normal range, indicating compensated Leydig cell dysfunction.

Young boys and adolescent males who receive standard dose cyclophosphamide (200mg/kg) as conditioning therapy for bone marrow transplantation appear to retain normal Leydig cell function, as evidenced by normal hormone profile and pubertal development in the majority of males[66]. Although the data are limited, Leydig cell function appears to be preserved in most males treated with the combination of busulphan and cyclophosphamide, despite damage to the seminiferous epithelium[67].

3.2. Radiotherapy-induced damage

3.2.1. Seminiferous epithelial damage

The seminiferous epithelium is very sensitive to damage from both radiotherapy and chemotherapy. The degree and permanency of radiotherapy-induced testicular damage depends on the treatment field, total dose and fractionation schedule (Table 3.2)[13-16,68]. Doses as low as 0.1-1.2 Gy damage dividing spermatogonia and disrupt cell morphology resulting in oligozoospermia[68,69]. Following low-dose, single fraction irradiation, complete recovery of spermatogenesis was observed 9-18 months following irradiation with 1 Gy, by 30 months following doses of 2-3 Gy and at 5 years or more in those treated with 4 Gy[68,69]. The seminiferous epithelium appears to be more susceptible to fractionation of radiotherapy, with doses greater than 1.2 Gy fractionated resulting in permanent azoospermia[15].

Table 3.2. Radiotherapy-induced damage to the reproductive tract

Site	Effect
Cranial irradiation	Hypogonadotrophic-hypogonadism
TBI/pelvic/testes	Seminiferous epithelium
	>1.2Gy – azoospermia
	0.1-1.1Gy- oligoozospermia
	Leydig cells
	>20 Gy – prepubertal
	>30 Gy – post-pubertal

In a large study, 463 males aged 26 (11-62) (mean (range)) years, received TBI, 10-15.75 Gy, as preparative treatment before BMT for haematological malignancies or aplastic anaemia[16]. Assessment of testicular function, at 3 (1-19) years post transplant demonstrated impaired testicular function, with normal defined as normal FSH, LH and testosterone concentrations with evidence of sperm production, in 83% (382 of 463) of subjects.

The effects of irradiation on testicular function were evaluated in 60 long-term survivors of childhood ALL. All patients received 18 or 24 Gy cranial or craniospinal irradiation, groups one and two, and the third group received 12 Gy abdominal irradiation in addition to craniospinal irradiation. Primary germ cell dysfunction, defined as elevated FSH and/or reduced testicular volume, was significantly associated with the field of radiotherapy. Of the patients who received additional abdominal irradiation, 55% demonstrated evidence of seminiferous epithelial dysfunction, in contrast to none of the patients treated with cranial irradiation alone. Interestingly, 17% of the group receiving craniospinal irradiation demonstrated evidence of primary germ cell damage, indicating that scatter irradiation exposure may also impair testicular function[13].

Gonadal function was assessed in 15 boys following testicular irradiation for childhood ALL. The dose to the testes was 12 Gy in 12 boys, 15 Gy in one boy and 24 Gy in the remaining two boys. Azoospermia was observed in all boys[70].

In a further study, testicular function was investigated in 21 boys, mean age 19.0 years, following total body irradiation and bone marrow transplantation for haematological malignancies at a mean age of 11.3 years, 15 of whom were prepubertal at time of treatment. All boys had reduced testicular volumes (mean 10.5ml) with elevated basal FSH concentrations, with normalisation in only one patient, indicating severe impairment of reproductive function following TBI[72].

3.2.2. Leydig cell damage

Leydig cells are more resistant to damage from radiotherapy than the seminiferous epithelium and progression through puberty with normal potency is common despite severe impairment of spermatogenesis. Susceptibility to radiation-induced Leydig cell damage appears to be inversely related to age, or sexual maturation, with greater damage following smaller doses in prepubertal boys[13,14,70-75].

Impact of radiotherapy on testicular function has been studied in children undergoing BMT for leukaemia. Seventeen prepubertal boys, aged less than 12 years, received hyperfractionated TBI (TD: 13.75-15 Gy) with a testicular boost of 4Gy in 16 patients and 12 Gy in the remaining patient. Before undergoing transplantation, all of the boys had received multi-agent chemotherapy, and for nine of them this had included cyclophosphamide. Of the 17 boys, median age 14 years (10.4-17.1), 14 (82%) entered puberty spontaneously, two were less than 12 years old and prepubertal, and the remaining subject, who had received a 12 Gy testicular boost, required androgen replacement therapy to induce puberty. Although overt Leydig

cell failure was rare, 36% of the subjects demonstrated compensated Leydig cell dysfunction. Furthermore, pubertal boys with raised plasma LH concentrations were younger at the time of BMT than pubertal boys with normal levels of LH[73]. Similar reports of increased vulnerability of the young Leydig cell to radiation-induced damage are reported in a number of studies[66,73-76]. Leydig cell function was evaluated in 41 men treated with total body irradiation (12 Gy fractionated or 10 Gy single dose), 6 Gy single dose total lymphoid irradiation or chemotherapy alone at median age 7.7 years (0.6-13.6). Leydig cell dysfunction was reported in 10 (24%) of the men, of whom 3 had complete Leydig cell failure[76]. Testicular irradiation with doses of greater than 20 Gy is associated with Leydig cell dysfunction in prepubertal boys while Leydig cell function is usually preserved up to 30 Gy in sexually mature males (Table 3.2)[73-76].

Pubertal development after TBI was investigated in 21 boys treated with allogeneic BMT for haematological malignancies at a mean age of 11.3 years. Fifteen of the boys were prepubertal at BMT. Normal development of secondary sexual characteristics was observed in 19 men. The remaining 2 men with hypogonadism had received an additional boost of testicular irradiation and required androgen supplementation. However, despite clinical evidence of intact Leydig cell function, and normal testosterone levels in all 19 men, LH levels were elevated in the majority of subjects indicating mild Leydig cell dysfunction[72].

3.3. Clinical impact of impaired testicular function

The clinical implications of frank Leydig cell failure and azoospermia are very clear. The implications of mild/subclinical Leydig cell insufficiency are, however, unclear. The clinical manifestations of Leydig cell dysfunction will inevitably depend upon the age of the child at the time of treatment. Loss of Leydig cell function before the onset of, or during puberty, will be associated with failure to enter puberty spontaneously or arrest of pubertal development. Cytotoxic insult following the development of normal secondary sexual characteristics will manifest clinically as erectile dysfunction, reduced libido, fatigue and mood changes, and biochemically will be associated with decreased bone mineral density, loss of muscle mass and other metabolic disturbances[17-19].

Leydig cell failure in the prepubertal child will require androgen supplementation for induction and progression of puberty and in the adult, testosterone replacement. Increasing evidence is emerging that compensated Leydig cell impairment may play an important role in bone mineral density and general well being. In the adult male, overt testosterone deficiency is associated with decreased energy level, poor libido, increased incidence of anxiety and depression, altered body composition and reduced bone mineral density (BMD). These symptoms if found in males following chemotherapy imply mild testosterone deficiency[17-19]. Limited data are available exploring the role of testosterone supplementation in men with compensated Leydig cell dysfunction. In a single blind randomised study, 35 men (mean age 40.9 years) with mild Leydig cell dysfunction, as defined by raised LH and low or low normal

testosterone level, were identified following treatment with cytotoxic chemotherapy for malignancy. Patients were randomised to receive 12 months treatment with transdermal testosterone or placebo patches. Testosterone levels increased significantly in the testosterone treated group compared with the placebo group and LH levels returned to normal values in 94% of subjects. However, there were no significant changes in bone mineral density, body composition or lipid profile, apart from a small reduction in LDL cholesterol. In terms of quality of life, the only perceived benefit was a marginal reduction in physical fatigue with no effects on mood or sexual function[77]. Testosterone supplementation for mild hypogonadism appears to be of limited clinical benefit but further studies are required to further our understanding and define the role of testosterone replacement therapy.

3.4. Progeny

Overall there are reassuring reports that there is no increased incidence of either congenital abnormalities or childhood malignancy in children born to long-term survivors of childhood cancer[78,79]. However these successful pregnancies mostly result from normally achieved conception. We do not know the consequences of circumventing the natural selection processes of normal sexual reproduction using assisted reproduction techniques (ART), nor the effects of ART on the complex cascade of precisely timed molecular interactions of early embryonic development. Continued surveillance of the progeny of survivors of childhood cancer remains essential[80].

3.4.1. Paternal risk to the offspring

The mutagenic potential of cancer therapy may confer a risk to the foetus conceived using gametes produced after cancer therapy, although current epidemiological data suggests that offspring of cancer survivors do not have an increased incidence of congenital abnormalities or cancer relative to the general population[78,79]. There is at least the hypothetical possibility of injection of abnormal spermatozoa or immature spermatogenic cells carrying abnormal genomic DNA with the potential to increase congenital and other abnormalities amongst offspring[80,81]. Studies in animals have shown that exposure of the male germ line to chemotherapy agents may disrupt spermatozoal DNA and result in deleterious effects on embryo development[82-84]. Awareness of the importance of sperm DNA integrity for accurate transmission of genetic material to the offspring has necessitated the development of new techniques to assess sperm characteristics in more detail[81]. It has become clear that men from subfertility clinic populations, with abnormalities of the conventional criteria of semen quality, also demonstrate elevated levels of damage to the genomic DNA in their gametes. Even amongst normal populations, sperm chromatin damage has been linked with impaired fecundity[85]. It has been shown that sperm DNA damage does not preclude pronucleus formation at ICSI, and that abnormal DNA within the male gamete is detectable in the early embryo[86]. Thus far, evidence on the safety of ICSI has been largely based upon its use in populations of men with deficits in spermatogenesis unrelated to potentially mutagenic cancer treatment. This evidence has been broadly reassuring concerning health risks to the offspring, although it is limited by the restricted length of follow-up currently available[87,88]. The quality of

spermatozoal DNA in long-term survivors of childhood cancer is investigated and the results are discussed in Chapter 4[53].

Successful pregnancies have been achieved using immature spermatogenic cells, which adds an unquantified risk to the foetus[89]. Fertilisation of oocytes with immature spermatogenic cells, such as round and elongated spermatids, which have not yet completed spermatogenesis, must be pursued with caution. The mechanism by which sperm precursor cells activate the oocyte at fertilisation is uncertain but it is speculated that suboptimal oocyte activation may confer poor fertilisation, implantation and high early abortion rates[89]. Spermatid transition into spermatozoa is characterised by salient changes in nuclear protein composition. The significance of circumventing these changes is uncertain[90]. Genetic imprinting plays an important role in embryogenesis and in processes leading to the development of paediatric cancers, including Wilms' tumour and embryonal rhabdomyosarcoma, and other human diseases[90]. Although the mechanism involved in genetic imprinting is uncertain, it is likely to involve differences in DNA methylation and requires careful consideration when embarking on germ cell maturation. Children born following assisted conception using spermatozoa and immature spermatogenic cells require long-term careful monitoring.

Chapter 4

Semen quality and spermatozoal DNA integrity in survivors of childhood cancer

4. Semen quality and spermatozoal DNA integrity in survivors of childhood cancer

4.1. Introduction

With continued improvement in survival from childhood cancer, such that 70% of patients are now long-term survivors, attention has focused on the lasting morbidity associated with radiation and chemotherapy treatment[2]. One of the most frequently encountered and psychologically traumatic late complications following treatment is infertility. Cytotoxic chemotherapy agents, particularly alkylating agents, may produce long-lasting or permanent damage to the seminiferous epithelium, resulting in oligozoospermia or azoospermia[4-9]. In addition, the seminiferous epithelium is very sensitive to radiotherapy and doses as low as 1.2 Gy may result in permanent sterility[15]. Recovery from surviving germ cells may occur but is unpredictable and often prolonged[10,11]. Leydig cells, with their slower rate of turnover, are more resistant to gonadotoxic therapy, resulting in preservation of androgen production even when patients are infertile[74].

Advances in techniques of assisted reproduction, and particularly intracytoplasmic sperm injection (ICSI), have provided a treatment option to enable men with oligozoospermia to achieve fatherhood[22,91]. Concerns have been raised about the safety of ICSI[92], particularly relating to the possibility that spermatozoa from men with impaired spermatogenesis may carry abnormal genetic information[80,86,93]. Although the best available data on the health of offspring following ICSI are broadly reassuring[87], there are no data on the health of offspring where the man's

deficit in semen quality is a consequence of potentially mutagenic treatment[94]. Studies in animals have shown that exposure of the male germ line to chemotherapy agents may disrupt spermatozoal DNA and result in deleterious effects on embryo development[82]. Awareness of the importance of sperm DNA integrity for accurate transmission of genetic material to the offspring has necessitated the development of new techniques to assess sperm characteristics in more detail[81].

The aim of this study was to investigate testicular function and semen quality in survivors of childhood cancer. The role of sexual maturation and endocrine parameters were investigated as potential markers of testicular function and spermatogenesis. Amongst patients who were not azoospermic, detailed assessment of semen characteristics was made including assessment of sperm DNA integrity.

4.2. Patients and Methods

The study was approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee and all patients gave written informed consent.

4.2.1. Patients

A review of the oncology database, at the Royal Hospital for Sick Children, Edinburgh, for all male survivors of childhood cancer, over the age of 16 years, identified 51 male survivors, median age of 22.2 years (range 16.5-35.2). Forty-five men were invited to participate in the study. Of the remaining six men, five had moved away from our region and one was on antidepressant medication and therefore excluded. Six men declined and six did not reply to the invitation. The 18 men who did not participate in the study were comparable for age, diagnoses, age at diagnosis, treatment regimens and disease free survival. Thirty-three men, aged 21.9 (16.5-35.2) years, participated in the study, a response rate of 73% of those invited, and an inclusion rate of 65% for the group as a whole. Of the 33 participants, median age (range) at diagnosis was 10.0 (2.2-16.9) years and disease free survival 11.6 (0.3-24.4) years. The underlying malignancies included acute lymphoblastic leukaemia (n=15), Hodgkin's disease (n=6), Ewing's sarcoma (n=5), non-Hodgkin's lymphoma (n=2), brain tumours (n=2), Wilms' tumour (n=1), osteosarcoma (n=1) and teratocarcinoma (n=1). A summary of the patients' diagnoses with details of the potentially gonadotoxic chemotherapy and radiotherapy received are summarised in Table 4.1. For each study patient, two age-matched controls (n=66) were recruited from a parallel survey of the reproductive health of normal volunteers. Volunteers

were recruited by means of advertisement in local media and through hospital outpatient clinics, and selected on the basis of the absence of any clinical evidence, on history or physical examination, of reproductive health problems.

4.2.2. Assessment of testicular function

Pubertal maturation was assessed according to the Tanner criteria and testicular volume (ml) was measured using the Prader Orchidometer[38]. The mean value of the two testes was taken to represent the subject's testicular volume. Venous blood samples were collected (20ml), and LH, FSH and testosterone levels were measured using automated immunoassay analyser (Bayer Immuno 1). A separate aliquot of the sample was centrifuged at 2000 x g for 10 minutes and serum stored at –20°C until assayed for inhibin B as previously described[95]. Semen samples were collected in a room adjacent to the laboratory, by masturbation into sterile wide mouthed non-toxic containers, following an abstinence period of at least 48 hours. These samples were analysed for ejaculate volume (ml), sperm concentration (x 10^6/ml), motility (%) and normal morphology (%) according to the protocols of the World Health Organisation (1999)[96]. Throughout the period of the study, the laboratory was subject to external quality control.

4.2.3. Assessment of sperm DNA integrity

DNA fragmentation in spermatozoa was measured using a modification of the method of terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) previously described by Sun *et al*[81]. An unselected

population of cells was obtained by mixing an aliquot of each sample with Biggers-Whitten-Whittingham (BWW) medium[97] containing 20nM N-(2-hydroxyethyl) piperazine-N'-(2-ethan sulfonic acid) (HEPES); Gibco, UK) and 0.3% human serum albumin solution (HAS) and centrifuged at 500 x g for 5 minutes. Following decanting of the supernatant, the spermatozoa pellet was resuspended in 2mls of phosphate buffered saline (PBS) (Sigma-Aldrich Co. Ltd Gillingham, UK) and centrifuged at 500 x g for 5 minutes. This step was repeated and the spermatozoa were subsequently fixed in 1% formaldehyde (Sigma-Aldrich Co. Ltd Gillingham, UK) in PBS for 60 minutes at room temperature. The fixed sperm concentration was adjusted to 20 x 10^6 cells/ml, using a Neubauer haemocytometer. The sample was centrifuged at 500 x g for 5 minutes and washed in PBS. The fixed sperm were resuspended in 100 µl prewash buffer (PWB) containing single strength One-Phor-All buffer (Amersham Pharmacia Biotech, Bucks., UK) and 0.1% Triton X-100 (Sigma-Aldrich Co. Ltd Gillingham, UK) for 15 minutes at room temperature. The sperm were spun out of the buffer at 500 x g for minutes and resuspended in 50 µl of TdT buffer containing 3 µM biotin-16-dUTP (Roche Diagnostics Ltd. Lewes, UK), 6 µM dATP (Amersham Pharmacia Biotech, Bucks., UK) and 1IU/µl of TdT enzyme (Amersham Pharmacia Biotech, Bucks., UK) and incubated at 37^0C for 60 minutes. After washing in PBS the fixed permeabilized sperm were resuspended in 100 µl of staining buffer consisting of 0.1%

Triton X-100 (in distilled water) and 1% streptavidin/fluorescin conjugate (Calbiochem-Novabiochem Ltd., Nottingham, UK) and incubated in the dark at 4^0C

for 30 minutes. The stained cells were spun at 500 x g for 5 minutes and resuspended in 500 µl PBS to give a concentration of approximately 1 x 10^6 cells/ml.

For negative controls, the enzyme terminal transferase was omitted from the reaction mixture. For positive controls, the samples were treated with 0.8IU/µl DNase I (Roche Diagnostics Ltd., Lewes, UK) for 15 minutes at room temperature, prior to the incubation with the TdT buffer.

4.2.4. Flow cytometry

The samples were analysed using a Epics XL flow cytometer (Beckman Coulter Corporation, Bucks., UK) with a 15mW argon ion laser operating at 488nm. Using the FL1 detector, green fluorescence was measured at 525nm. The flow rate during analysis was controlled at 200 events/sec, and 10 000 events were analysed in each sample. Light-scatter and fluorescence data were obtained at a fixed gain setting in the logarithmic mode. Debris were gated out based on the Forward Scatter versus Side Scatter dot plot by drawing a region enclosing the cell population of interest, and 10 000 events were collected. The data were processed using an IBM compatible computer installed with System II™ Version 1.0 software (Beckman Coulter Corporation, Bucks., UK). The percentage of labelled sperm in each sample was determined.

4.2.5. Testicular biopsy

Seven of the 10 men identified by semen analysis as being azoospermic were recruited into our study investigating suppression of the hypothalamic-pituitary-gonadal axis to restore spermatogenesis, described in chapter 6. As part of this latter study the seven men underwent testicular biopsy, under general anaesthetic, before commencing hormone therapy, which also served to exclude the diagnosis of obstructive azoospermia. The specimens were fixed in Bouins and stained with haemotoxylin and eosin and evaluated for the presence of spermatogonia.

4.2.6. Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Science (SPSS Inc., Chicago, Ill) version 10.0. Analysis of variance was performed on the endocrine data and ejaculate volume, the data common to all three groups. This involved Kruskal-Wallis tests for analysis of ejaculate volume (parametric) and one-way analysis of variance for the hormones (non-parametric). Where differences existed between the three groups pair-wise tests were performed to study differences between the groups. T-test and Mann-Whitney U-test were employed to compare the differences between patient groups and controls with regard to endocrine data and semen data respectively. For comparisons involving all three patient groups, three pair-wise two-sample tests were carried out and Bonferroni corrections were applied to the p-values. A difference of $p < 0.05$ was considered significant.

21	Osteosarcoma	-	-	-	-	-	-	None	33.75
22	Wilms'	-	-	-	-	-	-	None	37.1
23	ALL	-	-	-	-	-	NI	Cranium 24	42
24	Ewing's	-	-	-	138.6 (126)	-	-	None	53
25	ALL	-	-	-	-	0.78 (1.2) NI	1.3 (2) NI	Cranium 24	66.25
26	ALL	-	-	-	-	0.45 (0.6) NI	NI	Cranium 24	77
27	ALL	-	-	-	-	-	-	Cranium 18	85
28	Medulloblastoma	-	-	-	-	-	-	Cr/sp 55/35	94.83
29	ALL	-	-	-	-	-	-	Cranium 21	103.5
30	ALL	-	-	-	-	-	1.86 (2) NI	Cranium 18	113.25
31	Ewing's	-	-	-	144 (106)	-	-	None	125.25
32	ALL	-	-	-	-	-	-	Cranium 18	145
33	ALL	-	-	-	-	-	-	Cranium 18	230

Abbreviations: ALL: acute lymphoblastic leukaemia; HD: Hodgkin's disease; NHL: Non-Hodgkin's lymphoma; TD: total dose; Gy: Gray; Cr: cranium; TBI: total body irradiation; sp: spine; NI: patients received the agent but the dose received is indeterminable from the medical records.

4.3. Results

4.3.1. Patient characteristics

The childhood cancer survivor patient cohort and healthy volunteer control groups were comparable for age, smoking, alcohol consumption and abstinence (Table 4.2).

Table 4.2. Testicular function in long-term survivors of childhood cancer and healthy controls

Variable	Azoospermia (n=10)	Non-azoospermia (n=23)	Controls (n=66)
Patient characteristics			
Age: median (range) years	19.5 (16.5-25.3)	22.4 (17.6-35.2)	20.8 (18.0-36.3)
Smoking habits (%)	40	22	38
Alcohol consumption (%)			
0 (units/week)	10	26	8
<10	50	13	9
11-20	10	48	44
>21	30	13	39
Pubertal staging			
Tanner	5	5	5
Testicular volume (median, interquartile range)	10 (7.4-12)***	17.5 (15-21.25)	20 (15-23)

*** = P<0.001 for azoospermic group compared with other two groups

4.3.2. Semen analysis

Of the 33 patients, 10 (30.3%) were azoospermic (Table 4.1). Five of the azoospermic patients had received treatment for Hodgkin's disease with the alkylating agent chlorambucil, total dose, ≥340mg, ≥504mg/m^2, procarbazine ≥6.3g, ≥8.4g/m^2 and vinblastine ≥54mg, ≥72g/m^2, all of which are known to be gonadotoxic. Two of the azoospermic patients had been treated with ifosfamide (139.2, 84g/m^2; 165.6g, 87g/m^2) for Ewing's sarcoma, two had received total body irradiation (14.4Gy) and one had received direct testicular irradiation (24Gy). Of the 10 azoospermic patients, seven were prepubertal at diagnosis, providing cogent evidence that the prepubertal testis is not afforded protection from cytotoxic insult.

Six (18.2%) patients were oligoozospermia (sperm concentration <20 x 10^6/ml), with severe oligozoospermia (<2 x 10^6/ml) in one patient, while in contrast oligozoospermia was observed in only 4.5% of the control population (n=66). Only one of the six patients treated for Hodgkin's disease with an alkylating agent-based regimen showed preservation of spermatogenesis (sperm concentration: 4.55 x 10^6/ml). Three of the oligoozospermia patients had been treated with Medical Research Council Protocols, UKALL II, III and X, which consisted of combination chemotherapy, including, vincristine, prednisolone, 6-mercaptopurine methotrexate, cytarabine and cyclophosphamide. Oligozoospermia was observed in one of the five patients treated for Ewing's sarcoma, for whom treatment included both ifosfamide 132.6g, 102g/m^2, and cyclophosphamide 4.3g, 3.3g/m^2. The remaining oligoozospermia patient (sperm concentration: 0.55 x 10^6/ml) did not receive

treatment with agents expected to be gonadotoxic and the reason for impaired spermatogenesis is unclear.

Sperm concentration in the non-azoospermic group was significantly lower than the sperm concentration for the control population (95% CI: -67 to –15, $p<0.01$, Table 4.3, Figure 4.1). Nine (29%) of the subjects were asthenozoospermic (progressive motility <50%) compared with ten (15.2%) for the control group. Median progressive motility for the non-azoospermia group was significantly less than the control group (95% CI: -15.1 to –0.6, $p<0.05$, Table 4.3). The percentage of sperm with normal morphology was significantly less in the non-azoospermic group compared with the control population (95% CI: -4.6 to –1.3, $p<0.01$, Table 4.3). From our population of 33 male survivors of childhood cancer only 11 (33.3%) men had a normal semen analysis as defined by the World Health Organisation (1999) compared to 55 (83.3%) of the control group. There was no correlation between either age at diagnosis or time out from treatment and sperm concentration in this group of cancer survivors who were non-azoospermic ($p=0.109$ and $p=0.516$ respectively). Interestingly, there was a difference in ejaculate volume between the three groups ($p<0.001$). Ejaculate volume was significantly reduced in both the azoospermic and non-azoospermic groups, 1.9 (1.5-2.3) ml and 2.5 (2.1-3.5) ml, compared with the control population 3.4 (2.5-5.1) ml ($p=0.002$ and $p=0.006$ respectively).

Table 4.3. Semen quality in long-term survivors of childhood cancer and healthy controls

Semen parameters (median, interquartile range)	Azoospermia (n=10)	Non-azoospermia (n=23)	Controls (n=66)^	95% CI azoospermic vs. controls	95% CI non-azoospermic vs. azoospermic	95% CI non-azoospermic vs. controls
Abstinence (hours)	62 (40.5-137.8)	60.4 (36-82.5)	73.5 (59.3-91.5)			
Ejaculate volume (mls)	1.9 (1.5-2.3)	2.5 (2.1-3.5)	3.4 (2.5-5.1)	-2.7 to -0.7**	-1.5 to +0.2	-1.7 to -0.3**
Sperm concentration ($x10^6$/ml)	0	37.1 (19.7-89.9)	90.7 (50.5-121.5)			-67 to -15**
Progressive motility (%)	N/A	56.3 (44.4-64.7)	61.9 (55.5-69.1)			-15.1 to -0.6*
Normal morphology (%)	N/A	6.5 (3.7-7.6)	9.3 (6.3-11)			-4.6 to -1.3**
Tunel damage (%)	N/A	8.8 (5.1-12.6)	11.4 (7.2-16.3)			-5.3 to +0.2

* = $p<0.05$, ** = $p<0.01$, ^ = n=64 for Tunel assay

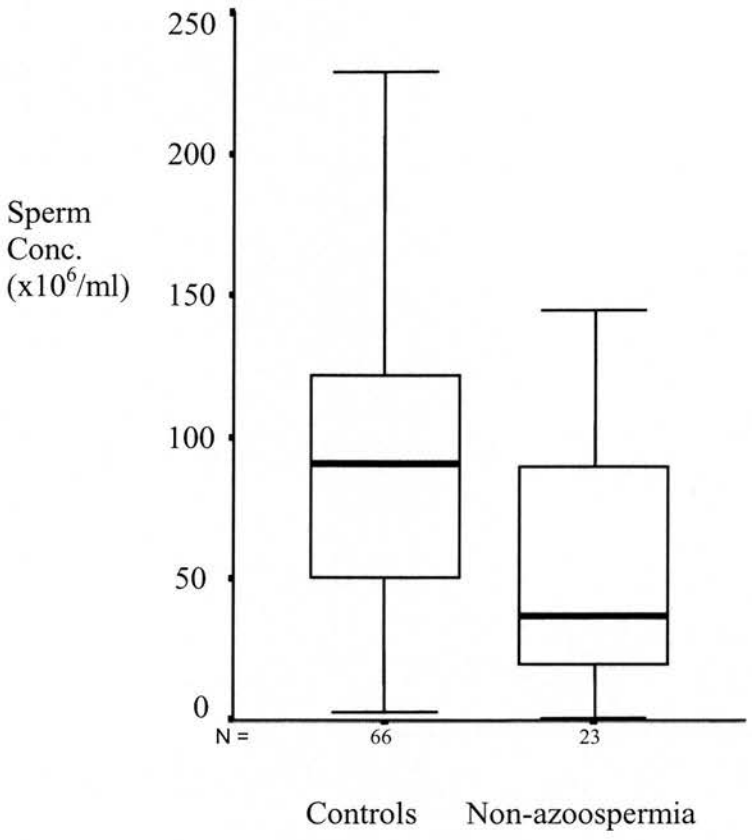


Figure 4.1. Sperm concentration in non-azoospermic long-term survivors of childhood cancer and in controls.

Median, IQR, and maximum and minimum values are shown.

4.3.3. Sperm DNA integrity studies

The integrity of the spermatozoa DNA was assessed using the TUNEL assay and the results are shown in Table 4.3. There was no statistically significant difference between DNA fragmentation in the non-azoospermic group (n=23) and the control population (n=64), 95% CI: -5.3 to +0.2, p= 0.06 (Table 4.3, Figure 4.2).

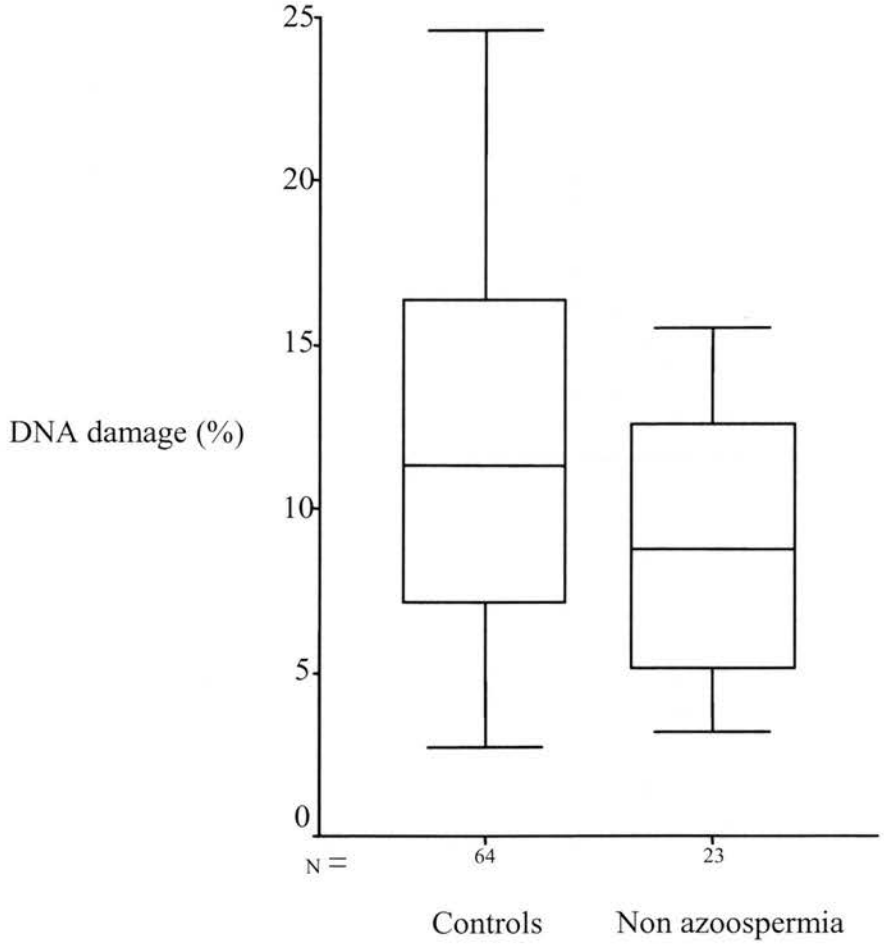


Figure 4.2. Sperm DNA integrity in long-term survivors of childhood cancer and in controls.

Median, IQR, and maximum and minimum values are shown.

4.3.4. Testicular Biopsy

Testicular biopsies from the seven of the 10 men with azoospermia on semen analysis demonstrated a Sertoli cell only picture in all cases thus excluding obstructive azoospermia (Figure 4.3a). Examination of the testicular tissue also demonstrated interstitial fibrosis, thickening of the basement membrane and atrophy of the seminiferous tubules, in contrast to the healthy seminiferous epithelium of a healthy adult man (Figure 4.3b).

4.3.5. Endocrine profile

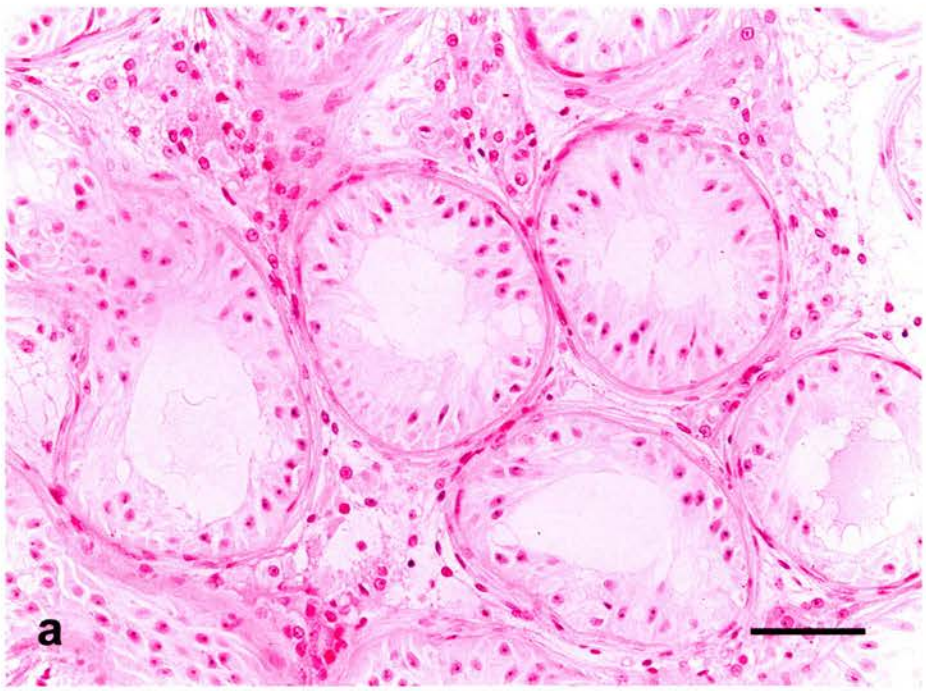
Analysis of variance for the endocrine measurements, FSH, LH, testosterone and inhibin B, demonstrated a significant difference for the FSH, LH and inhibin B values for the three groups ($p<0.001$) with no significant difference between the groups for testosterone levels. Pair-wise tests were subsequently performed to analyse the differences between the groups with Bonferroni corrections applied.

Basal serum FSH concentrations were significantly higher in the azoospermic group (n=10), (mean, ± standard error of mean (SEM)), 23.2 ±3.4 U/L compared with both the non-azoospermic (n=23) and control groups (n=53), 6.6 ±0.9 U/L (95% CI: -22.0 to –11.3, $p<0.001$) and 3.2 ±0.2 U/L (95% CI: -23.1 to –17.1, $p<0.001$, Figure 4.4a). Interestingly, the FSH concentrations were also significantly greater in the non-azoospermic group than in the control population (95% CI: –4.8 to –2.1, $p<0.001$), (Figure 4.4a). Inhibin B concentration was barely detectable in the azoospermic

group (n=10), 24.0 ±12.3 ng/L, which was significantly lower than in both the non-azoospermic (n=23) and control groups (n=53), 153.3 ±17.8 ng/L (95% CI: 61.2-190.5, $p<0.001$) and 222.9 ±8.7 ng/L (95% CI: 146.6 to 245.9, $p<0.001$) respectively (Figure 4.4b). Inhibin B concentrations were also significantly lower in the non-azoospermic group than the control group (95% CI: 34.6 to 106.3, $p<0.001$, Figure 4.4b).

Serum LH was significantly greater in both the non-azoospermic (n=23) and azoospermic groups (n=10), 6.0 ±0.8 and 9.2 ±1.3 U/L respectively, compared with the control group (n=52), 3.6 ±0.2 U/L (95% CI: -3.5 to –1.2 and -7.1 to - 4.2, $p<0.001$ in both cases, Figure 4.4c). Testosterone concentrations were within normal limits (10-30 nmol/L) in all 3 groups (azoospermic, non azoospermic and control groups, n=10, 23 and 53 respectively) with no significant differences between the groups (Figure 4.4d).

Figure. 4.3 Human testicular tissue stained with haematoxylin and eosin. Panel (a) shows testicular morphology from one of the study patients demonstrating complete absence of all germ cell types. A healthy adult male with normal seminiferous epithelium and abundant germ cell types is shown for comparison in panel (b). Scale bar denotes 50μm.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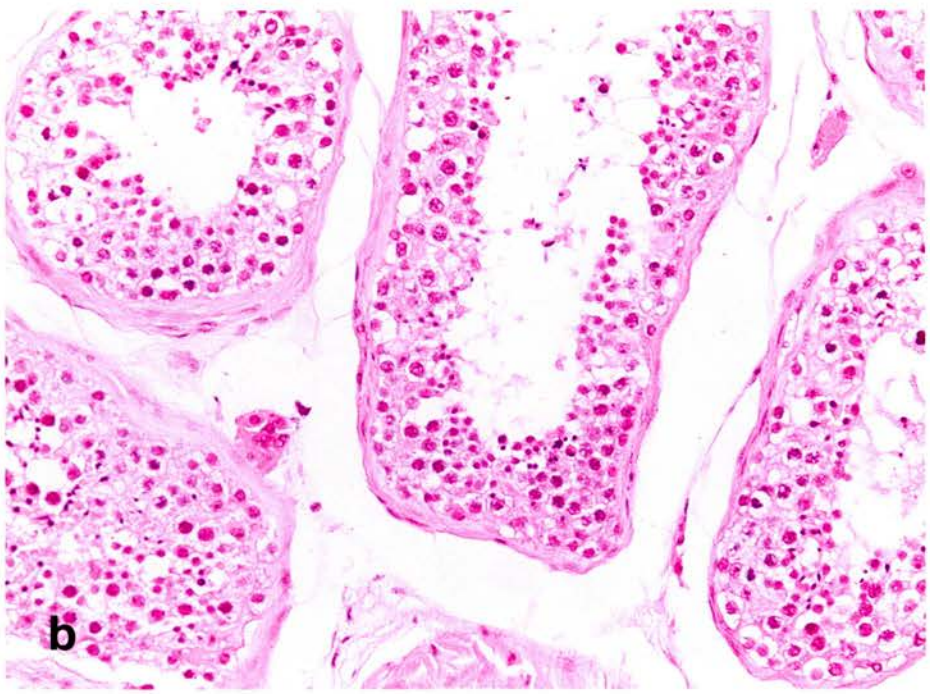
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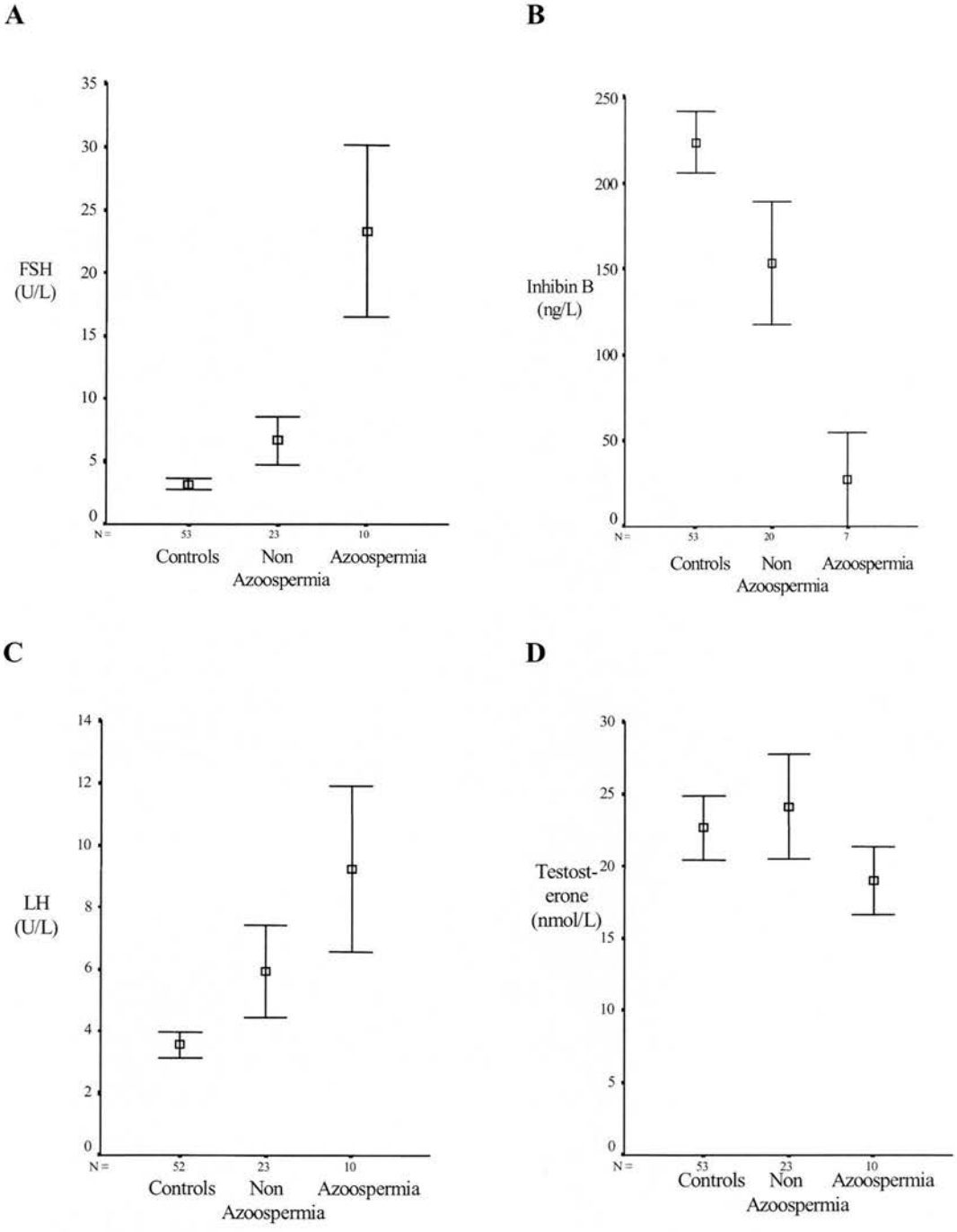


Figure 4.4. Comparison of values for follicle stimulating hormone (FSH) (A), inhibin B (B), luteinizing hormone (LH) (C), and testosterone (D) in the control group, and in the non-azoospermic and azoospermic cancer survivors.
Mean and SEM are shown. Reference ranges for FSH and LH are 1.5-9.0 U/L and for testosterone 10-30 nmol/L. There is no established reference range for inhibin B.

4.4. Discussion

We have shown that the treatment of childhood cancer is associated with a significant risk of subsequently impaired spermatogenesis, with 30.3% of this population being azoospermic and 18.2% being oligoozospermia. Moreover, in those men who do have surviving spermatogenesis after treatment, it is commonly compromised, with reductions being observed in ejaculate volume, sperm concentration, sperm motility and the proportion of morphologically normal sperm. Only 33.3% of this group of male childhood cancer survivors had completely normal semen quality by conventional criteria[96]. Correspondingly, this group also had lower inhibin B levels and higher FSH levels than the control population. Given that the control group was comparable in other respects, it is likely that the reduction in sperm number was directly attributable to the cytotoxic effects of cancer therapy. Reassuringly, the integrity of the genomic DNA carried by their gametes appears unaffected.

Fertility and sexual function are the principal life-style concerns in more than 80% of men successfully treated for cancer, yet, a substantial proportion of our survivors (27%) were reluctant to undergo an appraisal of their reproductive function, for reasons that are unknown. In addition to treatment induced infertility, it is well recognised that cancer survivors are less likely to have children for a number of reasons, including their inability to form relationships, fear of relapse of their disease, and the prospect of leaving their child parentless[98].

Cytotoxic treatment for childhood cancer should minimise unwanted side effects without compromising survival. Where there is equal efficacy between regimens the impact on reproductive function must be considered when devising the most appropriate therapy[50]. The present data confirm the sterilising effects of treatment for Hodgkin's disease with a standard regimen, ChlVPP (chlorambucil, vinblastine, procarbazine and prednisolone)[10,11]. The ABVD (adriamycin, bleomycin, vinblastine and dacarbazine) combination, which does not contain alkylating agents or procarbazine, has been shown to be significantly less gonadotoxic, resulting in temporary azoospermia in 33% of patients and oligozoospermia in 21%, with full recovery after 18 months reported in all patients[50]. Consequently, with the introduction of hybrid regimens, three cycles of ABVD with three cycles of ChlVPP or MOPP, (mechlorethamine, vincristine, procarbazine and prednisolone) gonadotoxicity may be significantly reduced.

Three of the sterile patients in our study had been treated with either 14.4 Gy fractionated total body irradiation (n=2) or 24 Gy testicular irradiation. Although recovery has been reported after a number of years, the high doses administered in these treatments make recovery unlikely[15]. Furthermore, as a prerequisite of recovery, some stem cells must survive, and for these three men, as indeed for all seven men biopsied, all germ cells (and thus including stem cells) were absent on testicular biopsy.

Ifosfamide, an analogue of cyclophosphamide, is potentially gonadotoxic but no data describing its effect on testicular function is available. Five of our cohort of patients had received treatment that included ifosfamide for Ewing's sarcoma. Only two of this group had a normal semen analysis (sperm concentrations: 53 and 125.25 x 10^6/ml), one was oligoozospermia (12.45 x 10^6/ml) and the remaining two patients were azoospermic. The three patients with sperm present in their ejaculate were 11.3 (9.41-11.73) years out from treatment, compared to the azoospermic patients who had completed treatment four and seven months previously. The two sterile patients were sexually mature at the time of diagnosis and had provided semen samples for cryopreservation before commencing cancer therapy, with sperm concentrations of 124 and 128 x10^6/ml. This is the first report of azoospermia in young men who had received ifosfamide containing regimens without any other potentially gonadotoxic agent. Recovery of testicular function with increasing time since treatment is a possible explanation. Continued, perhaps yearly, reassessment of semen analysis is warranted in azoospermic patients.

Normal semen quality, as defined by the World Health Organisation, stipulates that sperm concentration must be greater than 20 x 10^6/ml and progressive motility greater than 50%[96]. Using these criteria, only 33.3% (11 out of 33) of the survivors of childhood cancer had a normal semen analysis, compared with 83.3% (55 out of 66) in the control group. Our results are similar to those reported by Lopez Andreu *et al*[99].

Oligozoospermia was observed in six (18.2%) of the long-term survivor patients, compared with three (4.5%) in the control group. One of the patients, with oligozoospermia, had been treated with alkylating agent chemotherapy for Hodgkin's disease. Three of the oligozoospermia patients received treatment with standard protocols for ALL, which included cytarabine ($\geq$1g/m^2) and in one patient, cyclophosphamide (5.67g, 4.2g/m2). The doses of cytarabine and cyclophosphamide received by these three patients are significantly less than previously reported to be gonadotoxic doses[47] and may reflect individual susceptibility. Lendon *et al* studied testicular histology in 44 boys treated for ALL and found a severely depressed tubular fertility index (TFI), <40%, in 18 patients, indicative of seminiferous epithelial damage[62]. In a follow-up study by Wallace *et al*[45], semen analysis in seven of these patients with severe depression of TFI, 10.8 (5.5-15.9), (median, range) years off treatment, reported azoospermia in 4 of the patients and 'full' recovery of spermatogenesis in three patients (sperm concentration > 20 x 10^6/ml). From these studies, previous chemotherapy treatment with cyclophosphamide and cytarabine (>1g/m^2) impaired gonadal function, which improved with increasing time after treatment in some patients.

Inhibin B mediates non-steroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion[31,32]. Inhibin B secretion in the adult requires the presence of germ cells[31]. Inhibin B concentrations were barely detectable in the azoospermic patients, in whom the germ cells were destroyed, despite preservation of Sertoli cells, as confirmed on testicular biopsy.

This provides further evidence for the essential role of the germ cell-Sertoli cell interaction for the production of inhibin B and confirms the value of inhibin B as a non-invasive marker of spermatogenesis following cytotoxic therapy.

The sperm concentration in the non-azoospermic group was significantly less than that of the control population, 37.1 (19.7-89.9) x 10^6/ml compared with 90.7 (50.5-121.5) x 10^6/ml (p=0.002). Although azoospermia following gonadotoxic chemotherapy has been widely investigated there are few data about sperm concentrations in those patients in whom spermatogenesis is preserved. Rautonen *et al* reported sperm concentrations of median (range), 67 (0.01-425) x 10^6/ml in 27 patients treated for a variety of childhood haematological and solid malignancies[102]. Sperm concentrations of 20.4 (3.2-43) x 10^6/ml were reported in a small study cohort (n=4) treated for ALL (n=1) and non-Hodgkin's lymphoma (n=3) and 31 (4.5-100) x 10^6/ml in 8 long-term survivors of ALL, indicating a general trend towards a lower sperm concentration[102,103]. Given that intact spermatogenesis requires the presence of stem cells, which are capable of self-renewal in addition to differentiation, it is difficult to reconcile the discrepancy in sperm concentrations between the non-azoospermic and control groups. The non-azoospermic group clearly retains a population of intact stem cells capable of undergoing normal spermatogenesis, yet the surviving stem cells do not appear to repopulate the pool to produce sperm concentrations comparable to the control population. This could reflect a more complex picture highlighting the important role of Sertoli cells in supporting a finite number of stem cells. If there has been subtle damage to the seminiferous epithelium,

involving loss of Sertoli cells, the remaining Sertoli cells may already be functioning to full capacity. This is reflected in the subnormal inhibin B concentrations and corresponding elevation of serum FSH concentrations in the non-azoospermic group compared with the control population. This is analogous to data showing a decline in plasma inhibin B concentrations, directly in proportion to Sertoli cell numbers, following unilateral orchidectomy in rhesus monkeys[103]. In a physiological setting, where the negative feedback control system regulating the testes is operational, Sertoli cell number is the primary determinant of circulating inhibin B levels[103].

Ejaculate volume was significantly reduced in both the non-azoospermic and azoospermic groups compared with the control group, a finding which is unlikely to be attributable to retrograde ejaculation, obstruction, impaired autonomic innervation or incomplete specimen collection. Testosterone levels were normal in both groups of patients making testosterone deficiency an unlikely cause of decreased ejaculate volume. Damage to the prostate is unlikely, as only three of the patients had radiotherapy treatment involving the pelvis. Whether chemotherapy plays a role is uncertain. There is one other report of this finding by Rautonen *et al*[100]. In a study of 55 long-term survivors, treated with chemotherapy or radiotherapy for a variety of childhood malignancies, 18 (33%) were reported to have a low ejaculate volume, median (range), 2.0 (0.25-5.0)ml.

The mutagenic potential of cancer therapy may confer a risk to the foetus conceived using gametes produced after cancer therapy, although current epidemiological data

suggests that offspring of cancer survivors do not have an increased incidence of congenital abnormalities or cancer relative to the general population[78,79]. However, an important concern is that these results are largely based on offspring arising from natural conception and the consequences of circumventing the natural selection processes of fertilization involved by means of ICSI, are unknown[80]. There is at least the hypothetical possibility of injection of abnormal spermatozoa carrying abnormal genomic DNA with the potential to increase congenital and other abnormalities amongst offspring[81]. Techniques to evaluate sperm DNA integrity have been developed[81,93] and it is has become clear that men from subfertility clinic populations, with abnormalities of the conventional criteria of semen quality, also demonstrate elevated levels of damage to the genomic DNA in their gametes. Even amongst normal populations, sperm chromatin damage has been linked with impaired fecundity[85]. It has been shown that sperm DNA damage does not preclude pronucleus formation at ICSI[86], and that abnormal DNA within the male gamete is detectable in the early embryo[104]. Concern thus arises in the case of childhood cancer survivors, given that the capacity of ionising radiation and some chemicals to induce transmissible genetic damage in the germ cells of laboratory mammals has been clearly demonstrated[105]. Thus far, evidence on the safety of ICSI has been largely based upon its use in populations of men with deficits in spermatogenesis unrelated to potentially mutagenic cancer treatment. This evidence has been broadly reassuring concerning health risks to the offspring[87,88], although it is limited by restricted length of follow-up currently available. We have shown that although the conventional criteria of semen quality are frequently abnormal in long-term survivors of childhood

cancer, the sperm produced do not appear to carry a greater burden of damaged DNA. This observation goes some way to providing reassurance about the use of ICSI, which will circumvent the problems associated with severe oligozoospermia and asthenozoospermia, and offer cancer survivors the possibility of paternity in adulthood.

Chapter 5

Inhibin B as a marker of gonadotoxicity in prepubertal children treated for cancer

5. Inhibin B as a marker of gonadotoxicity in prepubertal children treated for cancer

5.1. Introduction

Traditionally, determining the impact of chemotherapy and radiotherapy on gonadal function has involved clinical assessment of pubertal development and semen analysis in males. Earlier detection of gonadal damage has been hampered by the lack of a sensitive marker of gonadal function in prepubertal children. In these children analysis of gonadotrophins is an unreliable predictor of gonadal damage because the hypothalamic-pituitary-gonadal axis is relatively quiescent. Inhibin B is a dimeric glycoprotein produced by the gonads, secreted from Sertoli cells in males[32,106]. In the adult male inhibin B plays an important role in spermatogenesis, mediated in part by inhibition of FSH secretion. There is increasing evidence reporting that the prepubertal gonads are not quiescent and have a low level of activity. The role of inhibin B as a marker of early gonadotoxic effects of chemotherapy was investigated in prepubertal children treated for cancer.

5.2. Patients and methods

5.2.1. Patients

Stored plasma was analysed from blood samples collected for previous prospective, longitudinal studies exploring the effects of chemotherapy on growth and bone turnover[107]. The original studies and their extension to this study were approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee.

The previous studies involved sequential recruitment of 29 boys, median age 4.5y (range 1.2 - 15.3y), with a variety of haematological and solid malignancies over a two year period. From this cohort were excluded patients for whom there was insufficient plasma stored pre- or during treatment (n = 8) and post-pubertal patients (n = 5). The study group consisted of 16 clinically prepubertal children with a median age (range) at diagnosis of 4.5y (1.2 - 12.8y) and underlying malignancies included acute lymphoblastic leukaemia (ALL, n = 6), neuroblastoma (n = 4), non-Hodgkin's lymphoma (n = 3), rhabdomyosarcoma (n = 1), osteosarcoma (n = 1) and Wilms' tumour (n = 1). Children with solid tumours (n = 10) were treated with multiagent chemotherapy regimens in accordance with United Kingdom Children's Cancer Study Group (UKCCSG) protocols. Children with ALL (n = 6) were treated with the MRC UKALL XI protocol. Blood samples had been collected from patients with solid malignancies (n = 10) before and during chemotherapy (median number of cycles: 7, range 4 - 14) and from patients with ALL (n = 6) pre-treatment and at the end of week 6 (after completion of induction and first intensification). In 7 of the 16 children treated for cancer, follow-up blood samples were also available between 1 and 6 months after completion of chemotherapy.

5.2.2. Methods

Following collection, blood samples were separated immediately and plasma was stored at -70°C until analysis. Dimeric inhibin B was measured in duplicate by a double antibody enzyme-linked immunosorbent assay, using a monoclonal antibody raised against a synthetic peptide from the βB subunit, combined with an antibody to

an inhibin α subunit sequence, as described[95,108]. The assay detection limit was 8 ng/L. Within and between assay coefficients of variation were 9.6% and 13.0% at 19 ng/L, 7.4% and 10.6% at 88 ng/L, and 8.4% and 10.2% at 233 ng/L respectively. FSH and LH were measured in duplicate by Delfia time-resolved immunofluorescence assays (Wallace, Milton Keynes, United Kingdom), standardised against the second International Reference Preparation of pituitary LH/FSH 78/549. Assay detection limits were 0.3 U/L for FSH and 0.2 U/L for LH. Between-assay CVs for FSH were 6.5% at 5.2 U/L, 3.5% at 10.4 U/L and 3.6% at 35.3 U/L, and for LH were 5.9% at 6.4 U/L, 5.5% at 11.3 U/L and 4.4% at 18.7 U/L. (These assays were carried as a service by the biochemistry department of the Royal Hospital for Sick Children, Edinburgh, under the guidance of Dr P. Crofton).

5.2.3. Data analysis

Inhibin B levels in the blood vary with age and sex. Data are presented both as absolute concentrations (ng/L) and as SD scores calculated for age and sex following log-transformation, based on our own published data[108]. Group results are presented as medians and ranges. Within-individual changes through time were evaluated by Wilcoxon matched pairs.

5.3. Results

Inhibin B levels were appropriate for age with no significant changes before, during and after treatment in all but one boy (Table 5.1)[108]. FSH and LH levels remained prepubertal in all patients pre-, during- and post- treatment (Table 5.1). Following completion of treatment, six of the seven boys showed little change in inhibin B (Figure 5.1). In one patient (aged 2yrs), inhibin B levels decreased to undetectable levels following completion of treatment with alkylating-agent based gonadotoxic chemotherapy (cyclophosphamide, 4.4 g/m^2, cis-platin 320 mg/m^2 and melphalan 200mg/m^2) with no post-treatment increase in gonadotrophins (FSH 0.8 U/L, LH <0.2 U/L).

Table 5.1. Median (range) of inhibin B, FSH and LH levels in boys before, during and after chemotherapy

	Before	During	After
N *	16 (10)	16 (10)	7 (6)
Inhibin B (ng/L)	93 (77, 244)	85 (58, 261)	94 (<8, 170)
Inhibin B (SDS)	-0.3 (-1.9, +1.8)	-0.62 (-1.8, +1.8)	-0.18 (<-7.0, +1.4)
FSH (U/L)	<0.3 (<0.3, 1.2)	<0.3 (<0.3, 0.8)	0.4 (<0.3, 2.6)
LH (U/L)	<0.2 (<0.2, 0.3)	<0.2 (<0.2, <0.2)	<0.2 (<0.2, 0.3)

* Total number of children with cancer (number with solid tumours).
SDS, SD score according to age and sex.

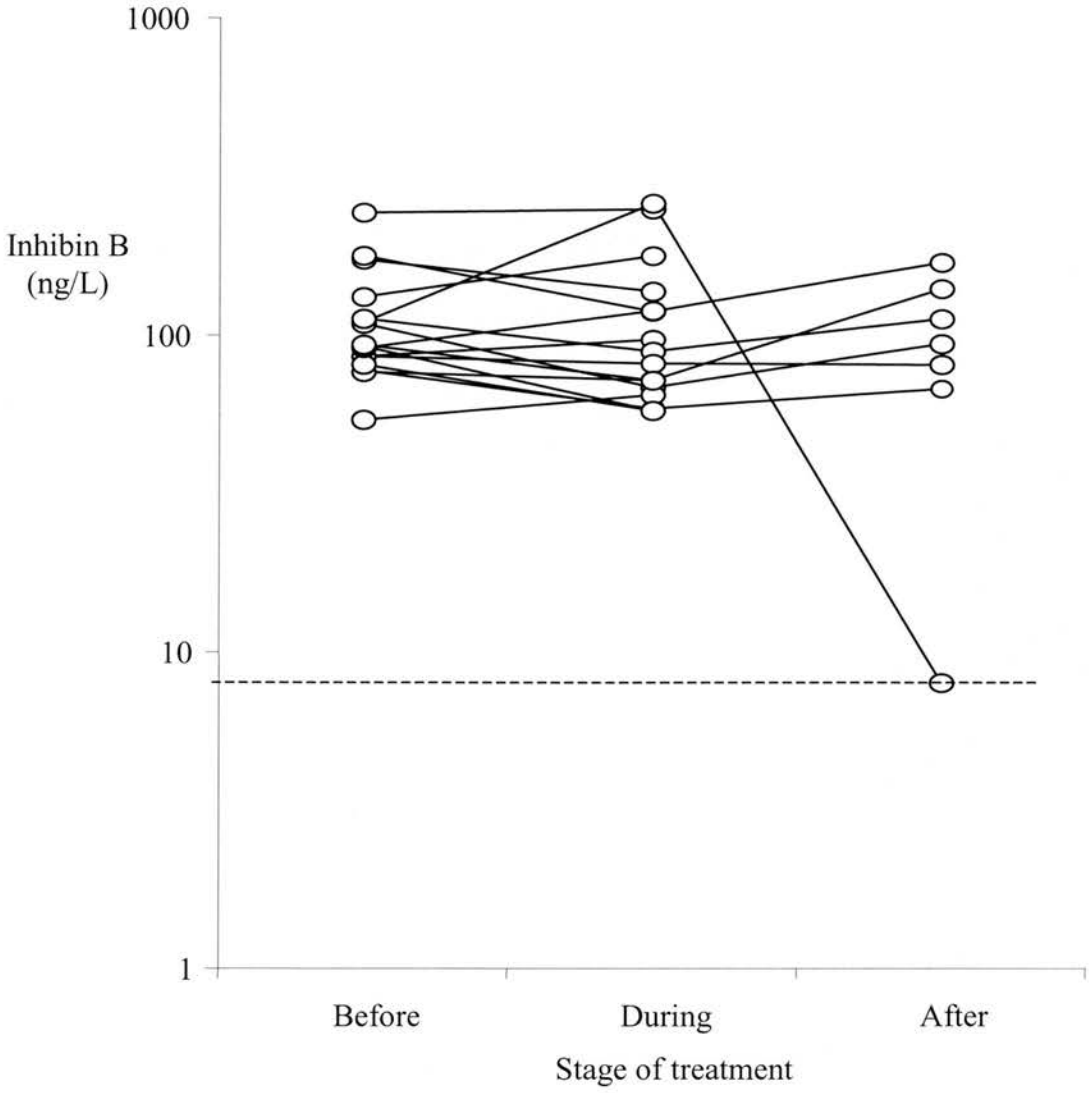


Figure 5.1 Inhibin B levels in individual boys with cancer before, during and after treatment. Inhibin B is plotted on a log scale. The dotted line represents the detection limit of the inhibin B assay.

5.4. Discussion

Inhibin B, produced from Sertoli cells, mediates non-steroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion[109]. Inhibin B secretion in the adult requires the presence of germ cells and in the prepubertal boy may reflect continuous Sertoli cell proliferation and functional activity together with maturation of early germ cells and spontaneous degradation. Men with complete absence of germ cells (Sertoli cell only syndrome, SCOS), severe hypospermatogenesis and spermatogonial and/or spermatocytic arrest have lower inhibin B levels and higher FSH levels than normozoospermic controls[110-112]. Gonadotoxic chemotherapy in men is associated with a decrease in circulating inhibin B during treatment[113]. A recent retrospective study of adult male survivors of childhood brain tumours found lower inhibin B and higher FSH levels in those treated with a combination of chemotherapy and radiotherapy compared with those treated with radiotherapy alone[114]. Another retrospective study of male survivors of childhood cancer found that previous treatment with alkylating agents was associated with low inhibin B and high FSH levels in postpubertal patients but normal inhibin B and variable FSH levels in pubertal patients[65]. Andersson and co-workers have demonstrated that, although men with SCOS have generally undetectable levels of inhibin B, prepubertal boys with acquired SCOS caused by irradiation/chemotherapy for ALL have normal prepubertal levels of inhibin B[115]. Follow-up post-pubertal samples from the same SCOS boys had undetectable inhibin B.

In this longitudinal study, boys with cancer had normal inhibin B levels before treatment started and these changed little during and after chemotherapy except for one boy with neuroblastoma treated with relatively high doses of known gonadotoxic agents, cyclophosphamide, cisplatin and melphalan[5,9,44,47] in whom inhibin B became undetectable following completion of treatment, suggestive of Sertoli cell damage. This may reflect damage to the seminiferous epithelium and long-term follow-up and monitoring of testicular function will be essential. The results show that inhibin B levels were normal in boys with ALL throughout treatment, including year 2 of continuing chemotherapy (data not shown), consistent with reports of normal testicular function and normal spermatogenesis in most male survivors of childhood ALL[45,64]. FSH and LH remained low or undetectable in all prepubertal boys with cancer before, during and after treatment, illustrating their insensitivity as markers of gonadal damage before the onset of puberty.

In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed deleterious effect. Inhibin B changed earlier and appeared to be a more sensitive marker of gonadal damage than FSH or LH. We conclude that inhibin B may be a useful addition to sensitive measurements of FSH in the evaluation of early gonadotoxic effects of chemotherapy in prepubertal children. This pilot study has not addressed the long-term implications for future fertility. Further studies are planned, combining inhibin B with FSH, LH and sex hormone measurements, to assess long-term reversibility

and delayed effects, particularly as the children approach and progress through puberty.

Chapter 6

Investigation of suppression of the hypothalamic-pituitary-gonadal axis to restore spermatogenesis in azoospermic men treated for childhood cancer

6. Investigation of suppression of the hypothalamic-pituitary-gonadal axis to restore spermatogenesis in azoospermic men treated for childhood cancer

6.1. Background to fertility preservation

Advances in assisted reproduction and increasing interest in gamete extraction and maturation have focused attention on preserving gonadal tissue from children before sterilising chemotherapy or radiotherapy, with the realistic expectation that future technologies will be able to utilize their immature gametes. The impetus for preserving gonadal tissue follows on the heels of pioneering experiments in ewes[116], together with media interest in the report of a successful autologous ovarian graft in a previously oophorectomized female. In addition live human births have been reported resulting from the transfer of embryos fertilized with immature spermatogenic cells[116-122]. Such issues have inevitably raised questions from parents and oncologists about their possible application in children undergoing cancer therapies[28,123].

6.1.1. Established practice

6.1.1.1. Cryopreservation of gametes

Potential strategies for preservation of male fertility are dependent upon the sexual maturity of the patient. The only established current clinical option for preservation of male fertility is cryopreservation of spermatozoa (Table 6.1). Spermatozoa are usually obtained from the ejaculate by masturbation but may be obtained using rectal electrostimulation techniques under anaesthetic. When it is not possible to obtain an ejaculate, sperm can be retrieved by epididymal aspiration or testicular biopsy in

sexually mature men. The onset of spermatogenesis, or spermarche, is difficult to determine. Spermarche is a mid-pubertal event, preceding the ability to produce an ejaculate, which occurs at a median age (range) of 13.4 (11.7-15.2) years when median testicular volume is 11.5 (4.7-19.6) mls[124]. Once spermarche is established, studies of serial urine specimens have shown that spermaturia is a variable, intermittent process throughout puberty[125-131]. Peripubertal males with spermaturia may identify a population of males who are able to produce sperm but not yet mature enough to produce a sample by masturbation. Surgical retrieval of spermatozoa may be considered for this group. In view of the clinical application of this investigation we proposed to determine the testicular volume at which spermaturia could reliably be expected to occur and to establish the number of days required to analyse the urine to detect 90% of those cases in whom spermarche had been achieved. Unfortunately, in a small pilot study the sperm concentration in urine from men immediately following ejaculation was very low making it highly unlikely that the detection rate in pubertal boys, where spermatogenesis is only just beginning, would be significant. Therefore, this study was not pursued.

Not infrequently, sperm produced by cancer patients at the time of diagnosis is of poor quality. With advances in assisted reproduction techniques, in particular intracytoplasmic sperm injection (ICSI), which involves the injection of a single spermatozoan directly into an ooctye, the problems of low numbers and poor motility may be cirvcumvented[21-23]. More recently a small number of pregnancies have been achieved with ICSI using immature spermatids and secondary spermatocytes extracted from testicular tissue in men with spermatogenic arrest[116-122].

6.1.2. Experimental strategies (Table 6.1)

Efforts to develop strategies to preserve fertile potential have focused largely on two areas, namely hormone manipulation and cryopreservation of testicular tissue.

6.1.2.1. Hormone manipulation

Following the hypothesis that the relatively quiescent prepubertal testis was less susceptible to the deleterious effects of chemotherapy and radiotherapy[132] it was postulated that inducing a prepubertal state by gonadal suppression would afford some protection to the testis during cytotoxic therapy. Although it is clear that cytotoxic therapy is gonadotoxic at all ages[13], the concept of manipulating the testicular milieu by gonadotrophin suppression has met with encouraging results in animal studies. We explored this approach in humans and the results from our study are described in this chapter.

6.1.2.2. Harvesting testicular tissue

For prepubertal boys, lacking in haploid gametes, there are no options currently available to preserve fertility, and any potential strategies must be considered entirely experimental. In theory testicular tissue could be removed before the start of treatment and cryopreserved either as a segment of tissue or as isolated germ cells. Following cure from his malignancy, the patient could have the frozen-thawed testicular tissue auto grafted to the testes. Alternatively, isolated germ cells could be re-implanted into the patient's own testes or these cells could be matured *in vitro* until they reached a stage sufficiently mature to achieve fertilization with ICSI.

The concept of testicular germ cell transplantation was pioneered by Brinster and colleagues in 1994[25]. Testicular germ cells isolated from mouse testis and transplanted into the testis of genetically or experimentally sterile mice initiated and sustained normal donor spermatogenesis, restoring fertility and producing healthy offspring[133]. Successful transplantation has also been shown in mouse recipients rendered sterile with the chemotherapeutic agent, busulphan. Interestingly these experiments also demonstrated both endogenous and donor spermatogenesis simultaneously, indicating that busulfan did not kill all the endogenous stem cells[25]. Subsequent studies have shown that testicular germ cell transplantation can occur through autologous, heterologous (mouse-to mouse, rat-to-rat) or xenologous (hamster, dog, rabbit or rat to mouse) transfer of cells. The site can be heterotopic, as graft to a non-gonadal site in the recipient, or homotopic when the cells are reintroduced into the testis[25-27,134,135]. In order to develop autologous germ cell transplantation in humans, techniques have to be developed by which human testicular germ cells can be isolated, stored and reintroduced into the testis.

Testicular tissue obtained by testicular biopsy has been successfully cryopreserved with subsequent isolation of spermatozoa from the thawed tissue and successfully used in ICSI[136,137]. These studies have only utilised spermatozoa or immature spermatogenic cells retrieved from tissue post thaw and it may be that survival of spermatogonial germ cells post-thaw is poor. Furthermore as there is a more than theoretical risk of transplanting tumour cells, it is likely that clinical practice will involve enzymatic digestion and isolation of germ cells before cryopreservation rather than autografting testicular tissue.

Transplantation in rodent models has involved only semi-purified cell populations of germ cells and somatic cells. For human application, purified stem cell populations would be necessary to ensure that no malignant cells were transferred. The term 'stem cell' is a functional description, given that there are no morphological, antigenic or biochemical criteria by which to identify these cells *in vivo* or *in vitro*. Spermatogonial transplantation is the only method at present by which stem cell presence can be authenticated. In mice LacZ encodes for the enzyme B-galactosidase, which can be demonstrated histochemically as an intracellular blue reaction product. Effective purification will demand the development of specific antibody probes to differentiate stem cells from other cells. Studies have shown that a number of cell surface antigens are expressed on stem cells, including alpha-6, beta-1 integrin and c-kit, which may enable enrichment using magnetic cell sorting[138]. The major limitation for human application is that these antigens are shared with other progenitor cells including haematopoietic cells, creating inherent problems in cancer patients.

The process of spermatogenesis begins in puberty although a number of immature spermatogenic cells are described in the prepubertal testis. It is believed that 10^4 germ cells contain as few as 2 stem cell spermatogonia capable of self-renewal. With the average number of germ cells in the testis estimated to range between 13-83 x 10^6 during childhood, the relatively small numbers of stem cell spermatogonia available poses a challenge for the process of stem cell isolation[35]. Consequently, there is a need to develop an *in vitro* culture system to augment stem cell numbers following harvesting and isolation. The feasibility of enrichment has been shown by Nagano *et al* who have

maintained mouse stem cells in culture for up to 4 months and the cultured cells initiated successful spermatogenesis following transplantation[139].

Cryopreservation of spermatozoa and fertilisation following thawing are well established. Application of this approach to stem cell spermatogonia will require modification and optimisation of freezing procedures, taking into consideration the inherent biological differences between the immature diploid stem cells and mature gametes[140]. There is thus a need for studies to devise cryopreservation regimens that are applicable to stem cells from the pre-pubertal testis.

6.1.2.2.1. Future use of cryopreserved testicular tissue

The future use of testicular germ cells is likely to involve either maturation of the germ cells *in vitro* for use with ICSI or transplantation. Ideally transplantation would involve injecting preparations of purified germ cells into the testis with restoration of natural fertility. *In vitro* maturation techniques, although still very much in their infancy, would have the advantage of eliminating any risk of transplanting harvested malignant cells back into the patient. Creating an environment *in vitro* to stimulate germ cell maturation and differentiation into spermatozoa may be the only option for a number of patients in whom cancer therapy damage has been so extensive that the supporting Sertoli cells would be unable to support spermatogenesis. Attempts to cultivate male germ cells in vitro have shown that germ cells can survive several months in culture and are likely to be undergoing cell division[141]. Tesarik reported *in vitro* spermatogenesis and healthy offspring using ICSI. However, the maturation process involved in vitro maturation of late stages of spermatogenesis rather than development from early germ cells[141,142].

A number of studies have concentrated on developing the most efficient technique for infusing the stem cells into the testis. The simplest and most effective method of filling the seminiferous tubules *in vitro* has proved to be by injection into the rete testis of bull, monkey and man, under ultrasound guidance[143]. These experiments involved injection into partially involuted or prepubertal testes. This eliminates the problems associated with backpressure of fluid in fully active testes, which would otherwise block the infusion of the cell suspension into the tubules. Successful *in vivo* rete injections have been performed on cynomologus monkeys treated with GnRH antagonist. Injecting the cells into the rete allows a much larger volume to be infused in contrast to the micro-injections involved in re-infusing directly into the seminiferous tubules[143]. Further work is required to perfect this technique for human application.

Immature testicular tissue has been shown to grow and differentiate when grafted into another species[26]. This provides an additional strategy for conserving the male germ line and circumvents the risk of reintroducing malignant cells. Clearly this technique is unlikely to be ethically acceptable and is compromised by the risk of interspecies transfer of potentially pathogenic micro-organisms.

Table 6.1. Clinical and experimental strategies for preservation of male reproductive function in children undergoing treatment for cancer

Clinical practice	Experimental strategies
Sperm banking	Cryopreservation of testicular tissue
- Ejaculation	Gonadotrophin suppression
- Rectal electrostimulation	
- Testicular/epididymal aspiration	

6.2. Introduction

A number of approaches to preserving fertility have been investigated based on the idea that suppression of spermatogenesis might protect the normally rapidly dividing germ cell population from damage. Suppression of the rat hypothalamic-pituitary-gonadal (H-P-G) axis by administration of the gonadotrophin releasing hormone (GnRH) analogue, goserelin, before and during chemotherapy with procarbazine, enhanced recovery of spermatogenesis[144]. Similarly, protection of spermatogenesis in rats subjected to treatment with procarbazine, cyclophosphamide and radiotherapy has been demonstrated using a number of hormones including testosterone alone[145] or in combination with oestrogen[146], GnRH analogues in combination with testosterone[147] or the anti-androgen flutamide[148,149].

Furthermore, recovery from spermatogenic damage in rats induced by radiotherapy or procarbazine treatment has been shown to be enhanced by treatment with GnRH analogues or testosterone even when administered after the gonadotoxic agent[147,150,151]. The mechanisms by which such hormonal manipulation offers protection or enhancement of recovery of spermatogenesis are unclear. Hormonal analysis following irradiation in rats has shown a dramatic increase in intratesticular testosterone levels and it has been postulated that suppression of the H-P-G axis promotes multiplication and differentiation of spermatogonia by lowering testosterone concentrations within the testis[150]. While there is significant evidence for the success of protection/recovery strategies in rats, clinical studies in man to date have been unsuccessful[152-155] and there have been no trials investigating the effects of post-gonadotoxic hormonal suppression. The present study has investigated whether suppression of the H-P-G axis in men

rendered azoospermic by treatment for childhood cancer might restore spermatogenesis, using both semen analysis and testicular biopsy as end points[156]. There is little information available on testicular histology in survivors of childhood cancer. To help further our understanding of the impact of sterilizing cancer therapy on testicular function we studied testicular histology in depth using immunohistochemical techniques.

6.3. Patients and Methods

The study was approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee and all patients gave written informed consent.

6.3.1. Patients

A review of the oncology database at the Royal Hospital for Sick Children, Edinburgh for men rendered azoospermic secondary to treatment for childhood cancer, identified seven men age 22.2 (18-25.3), median (range) years. These men formed part of the group of ten men with azoospermia identified in our studies assessing semen analysis and spermatozoal DNA integrity in survivors of childhood cancer (Chapter 4). The remaining three men were not identified until after the commencement of the hormone study. The men were invited to participate in the study regardless of the underlying malignancy or cytotoxic therapy and all seven accepted. The three men not identified early enough to be invited into the study, did not differ in terms of underlying diagnosis and severity of treatment. For the seven study patients, the median age at original diagnosis was 10.4 (4.4-13.3) years with a disease free survival of 8.4 (3.3-14.7) years. The underlying malignancies were acute lymphoblastic leukaemia (n=2), Hodgkin's disease (n=4) and non-Hodgkin's lymphoma (n=1). A summary of the patients' diagnoses with details of the gonadotoxic chemotherapy and radiotherapy received is given in Table 6.2. Clinical assessment and routine haematological and biochemical assessment was performed on each patient to ensure that there was no evidence of disease relapse or second primary malignancy, or other reason likely to impair spermatogenesis. None of the patients had a family history of impaired spermatogenesis.

Table 6.2. Patient diagnoses and their exposure to gonadotoxic treatment.

Patient	Diagnosis	Chlorambucil TDg(gm^{-2})	Procarbazine TDg(gm^{-2})	Vinblastine TDg(gm^{-2})	Cytarabine TDg(gm^{-2})	Radiotherapy TD(Gy)	Sperm concentration (10^6/ml)
1	ALL	-	-	-	2.76(2)	Cr/TBI 24/14.4	0
2	ALL/testis relapse	-	-	-	2.93(2)	Cr/testis 18/24	0
3	HD	0.9 (0.67)	16.8 (11.2)	134 (96)	-	None	0
4	HD	0.5 (0.504)	8.4 (8.4)	79.2 (72)	-	None	0
5	HD	0.5 (0.504)	10 (8.4)	86.4 (72)	-	Upper mantle 30	0
6	HD	0.34 (0.504)	6.3 (8.4)	54 (72)	-	Medias/neck 35	0
7	B-cell NHL	-	-	-	2.2 (2)	Cr/TBI 6/14.4	0

Abbreviations: ALL: acute lymphoblastic leukaemia; HD: Hodgkin's disease; NHL: Non-Hodgkin's lymphoma; TD: total dose; Gy: Gray; Cr: cranium; TBI: total body irradiation.

6.3.2. Assessment of testicular function

Pubertal maturation was assessed according to the Tanner criteria and testicular volume (ml) was measured using a Prader Orchidometer[38]. The mean value of the two testes was taken to represent the subject's testicular volume. Venous blood samples were collected (20ml), and LH, FSH and testosterone levels were measured using an automated immunoassay analyser (Bayer Immuno 1). Inhibin B was measured as previously described[95], with the limit of assay sensitivity being 7.8pg/ml. Semen samples were collected in a room adjacent to the laboratory, by masturbation into sterile wide mouthed non-toxic containers, following an abstinence period of at least 48 hours. Samples were centrifuged at 3000 x g for 30 minutes and the pellet examined to confirm azoospermia[96]. Seminal plasma was stored at –70°C until assayed for inhibin B[65]. Testicular biopsy under general anaesthetic was undertaken on all patients at the start of the study to exclude obstructive azoospermia. The specimens were fixed in Bouins fixative and after routine processing and paraffin embedding, sections were cut at 5μm and examined. A second biopsy of the same testis was performed at the end of the study.

6.3.3. Hypothalamic-pituitary-gonadal axis suppression

All men underwent a period of suppression of the H-P-G axis, designed to induce hypogonadotrophic hypogonadism with reduced intratesticular testosterone levels for a period of approximately 24 weeks, followed by a recovery period of 24 weeks. Following testicular biopsy, subjects were administered depot medroxyprogesterone

acetate (DMPA, 300mg i.m.: Pharmacia and Upjohn, Milton Keynes, UK) and testosterone pellets (4x 200mg s.c., NV Organon, Oss, The Netherlands). Administration of DMPA was repeated 12 weeks later. Subjects were reviewed at 6 weekly intervals throughout the 48 weeks of the study for clinical assessment, blood sampling and semen analysis.

6.3.4. Immunohistochemistry of testicular tissue

The objective of the immunohistochemical analysis was to investigate whether or not any germ cells, in any developmental stage, were present in the testes of patients before or after H-P-G suppressive treatment. This was achieved using immunoexpression of the MAGE-57B antigen and androgen receptor (AR). The MAGE-57B antigen is expressed in early germ cells, strongly in spermatogonia and weakly in early spermatocytes[157]. Androgen receptor is expressed in the nuclei of all Sertoli cells but not in the nuclei of any germ cells that might be present[158]. In addition, detailed evaluation of the extent of testicular damage was assessed by immunoexpression of androgen receptor (AR), specific for Sertoli, Leydig and peritubular cells: inhibin α and βB subunits, which localize to Sertoli and Leydig cells, and Sertoli cells respectively: expression of steroidogenic enzymes 3β-HSD and 17α-hyroxylase as markers of Leydig cell function.

Unless otherwise stated, all incubations were undertaken at room temperature. Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed in

water. A temperature-induced antigen retrieval step was required for androgen receptor, inhibin α- and β-B and 3β-HSD antigens. The sections were pressure cooked in 0.01M citrate buffer for androgen receptor, inhibin α- and 3β-HSD or 0.01M glycine buffer for inhibin β-B, pH 6.0 for 5 minutes at full pressure, allowed to stand for 20 minutes, cooled in running tap water and washed twice in (5 minutes each wash) in Tris-buffered saline (TBS: 0.05 mol Tris-HCL, pH 7.4, 0.85% (w/v) NaCl). Endogenous peroxidase activity was then blocked by immersing sections in 3% (v/v) H_2O_2 in methanol (both from BDH Laboratory Supplies, Poole) for 30 minutes, followed by two 5 minute washes in TBS. Sections were incubated for 30 minutes with the appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA: Sigma) to block non-specific binding sites. Normal swine serum (NSS) was used for androgen receptor and 3β-HSD; normal goat serum (NGS) for 17α-OH and normal rabbit serum (NRS) for inhibin α- and β-B and MAGE-57B (from Diagnostics Scotland, Carluke). Primary antibodies were added to the sections at the appropriate dilution in either NSS-TBS-BSA (for androgen receptor 1:2000: AR N-20, Santa Cruz Biotechnology SC0816; for 17 α–hydroxylase 1:2000, from, for 3β-HSD 1:2000, gifted from Professor I Mason), NRS-TBS-BSA (for inhibin α- 1:1000: 173.9K; inhibin β-B 1:6000, 12/13, both gifted from Professor N Groome, Oxford Brookes University; MAGE-57B: 1:50, gifted from G Spagnoli) and incubated overnight at 4°C in a humidified chamber. The sections were washed twice in TBS and then incubated for 30 minutes with anti-rabbit or horseradish peroxidase-labelled polymer (EnVision: DAKO, Ely) for

androgen receptor, 3β-HSD and 17α-OH or anti-mouse for inhibin α- and β-B and MAGE-57B. Sections were washed twice (5 minutes each) in TBS and immunostaining was developed using Liquid DAB (DAKO) until staining was optimal, when the reaction was stopped by immersing sections in distilled water. The sections were counter-stained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody.

Immunostained sections were examined and a mean of 132 (range 40-252) tubular cross-sections were counted for each specimen. The sections were photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, UK) fitted with a digital camera (Kodak DCS330: Eastman Kodak, Rochester, NY, USA). Captured images were stored on a computer (G4; Apple Computer Inc., Cupertino, CA) and compiled using Photoshop 5.0 before printing using an Epson Stylus 870 colour printer (Seiko Epson Corp., Nagano, Japan).

6.3.5. Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Science (SPSS Inc., Chicago, III) version 10.0. For each individual patient hormone concentrations at the beginning and end of the study were compared using t-tests and a $p < 0.05$ was considered significant.

6.4. Results

6.4.1. Clinical features

All seven men were Tanner stage V with reduced testicular volumes of 11 (5-12) ml, mean (range) (Table 6.3). There were no changes in clinical features during the study and no changes in testicular volume.

6.4.2. Hypothalamic-pituitary-gonadal axis suppression

MPA/testosterone treatment was well tolerated and there were no adverse effects. One man reported increased libido during the first 12 weeks of the study. Pre-treatment serum FSH levels were elevated, 22.4±4.4 U/L (mean±SEM, reference range: 1.5-9 U/L), in keeping with damage to the seminiferous epithelium (Table 6.3). Serum LH concentrations pre-treatment were 9.0±1.8 U/L (reference range: 1.5-9 U/L) and testosterone 17.9±1.5 nmol/L (reference range: 10-30 nmol/L), indicating compensated Leydig cell dysfunction (Table 6.3). Serum and seminal plasma inhibin B concentrations were barely detectable in all but one subject (subject no. 1: 35 ng/L and 770 ng/L respectively; Table 6.3).

FSH was suppressed to undetectable concentrations during MPA/testosterone treatment for 12 weeks, and remained partially suppressed during the subsequent 12 weeks (Figure 6.1). Thereafter, there was a gradual rise by weeks 42-48 to 19.5 ±3.6 U/L, which was not significantly different from pre-treatment concentrations. LH showed a similar pattern to FSH, with suppression to undetectable concentrations for

12 weeks, followed by gradual recovery to 8.9 ±1.6 U/L at 48 weeks (Figure 6.1b). There was no statistically significant difference between LH concentrations pre-treatment and at 48 weeks.

Conversely, testosterone concentrations rose initially following MPA/testosterone administration to a peak of 29.7 ±1.9 nmol/L at 6 weeks, close to the upper limit of the normal range. This was followed by a gradual decline to a nadir of 10.2 ±0.2 nmol/L at 30 weeks, with a subsequent slight rise to 13.8 ±1.9 nmol/L at weeks 42-48. The latter was not significantly different from the pre-treatment concentration.

Serum inhibin B concentrations increased from 35 ng/L to 60.9 ng/L at 12 weeks in the subject with the highest inhibin B pre-treatment, and rose from <15ng/L to low but detectable concentrations (range 16- 35 ng/L) in the 5 other subjects (venous serum reference range: mean 257, 95%CI 231-284ng/L). Serum inhibin B concentrations fell toward the end of the study, becoming undetectable in all subjects other than the individual with the highest pre-treatment concentration at weeks 42-48. This same individual was the only subject with readily detectable seminal plasma inhibin B concentration pre-treatment (770 ng/L, vs <20 ng/L in the others, seminal plasma reference range: mean 2279, 95% CI 698-3864ng/L)[32]. Seminal plasma inhibin B was not determined during MPA/testosterone treatment as the volume of the ejaculate was insufficient. In all subjects seminal plasma inhibin B concentrations were undetectable at the end of the study.

Table 6.3. Patient characteristics before hypothalamic-pituitary-gonadal suppression

Patient	Tanner stage	Testicular volume (ml) (Adult >12ml)	FSH (U/L) (ref range: 1.5-9)	LH (U/L) (ref range: 1.5-9)	Testosterone (nmol/L) (ref range: 10-30)	Inhibin B (venous) (ng/L)	Inhibin B (semen) (ng/L)
1	5	12	18.2	6.6	21.9	35.0	770
2	5	5	45.1	19	17.7	15.5	27.7
3	5	10	23.9	9.3	21.5	<7.8	14.2
4	5	10	23	8	20.5	<7.8	8.2
5	5	5.5	25.2	8.8	10.2	<7.8	<7.8
6	5	8	9.8	6.3	16.6	12.2	N/O
7	5	12	11.6	4.9	17.2	N/O	<7.8
		11	**22.4 (4.4)**	**9.0 (1.8)**	**17.9 (1.5)**		

Values in bold: mean (standard error of the mean)N/O: values not obtained

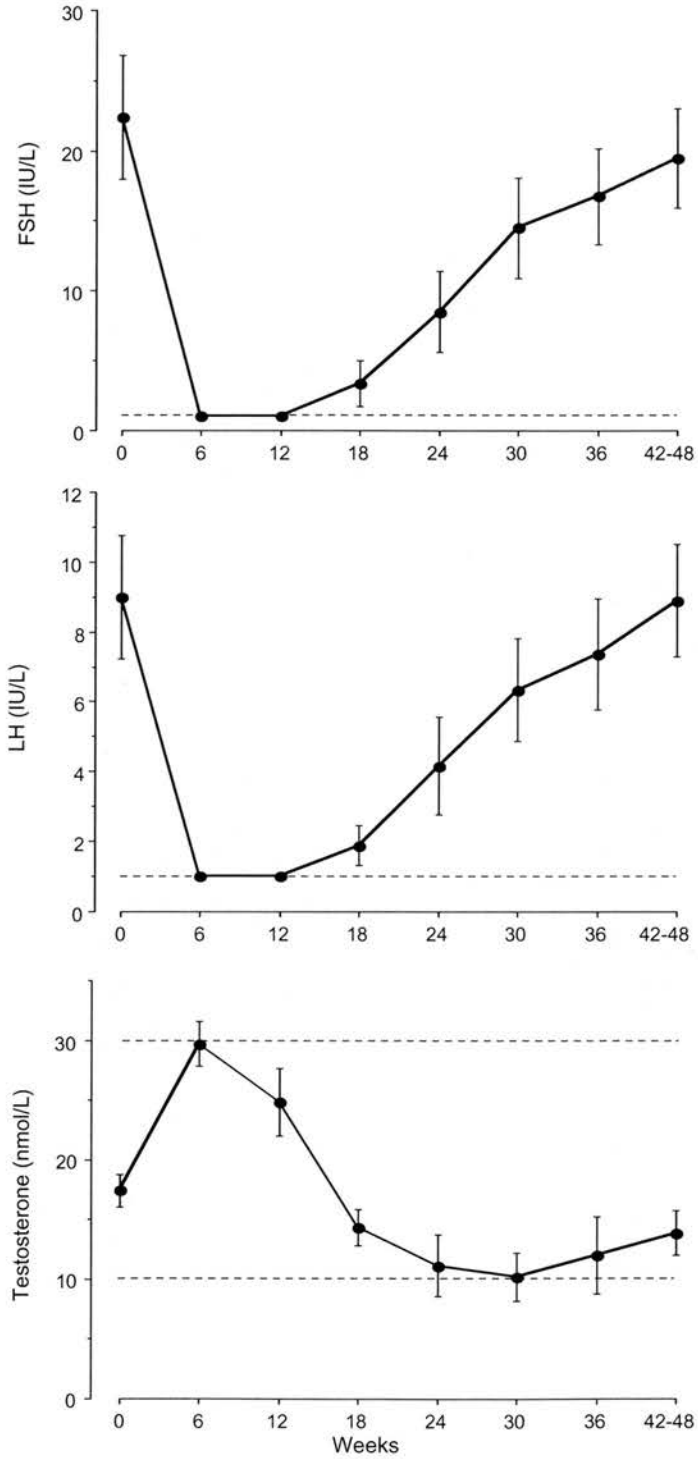


Figure 6.1. Serum concentrations of FSH, LH and testosterone in the seven patients before (time 0) and during combined treatment with medroxyprogesterone acetate and testosterone.
Values are mean ± SEM. Dashed lines show the limit of the assay sensitivity (FSH, LH) or the upper and lower limits of the normal range (testosterone). Hormone levels at 42-48 weeks are not significantly different from pre-treatment (time 0) values in each instance.

6.4.3. Semen analysis

All men remained azoospermic throughout the study.

6.4.4. Testicular tissue

6.4.4.1. Light microscopy

Examination of testicular tissue pre- and post H-P-G axis suppression demonstrated interstitial fibrosis, thickening of the basement membrane and atrophy of the seminiferous tubules with complete absence of all germ cells, in contrast to the abundant different germ cell types in the normal seminiferous epithelium of a healthy adult man (Figure 6.2). This was representative of all seven cancer survivors.

6.4.4.2. Immunocytochemistry

Androgen receptor (AR)

The nuclei of all cells within the seminiferous epithelium of all patients immunoexpressed AR (Figure 6.3(a) – brown staining), and this was comparable before MPA/testosterone treatment and at the end of the study (Figure 6.3(b)), in contrast to AR negative germ cells evident in the testis of a healthy adult man (Figure 6.3(c)). This finding suggested that only Sertoli cells were present within the tubules of the cancer patients. Using the methodology applied in the current study, all Sertoli cells in the human testis immunostained for AR with similar intensity[158], although application of modified methods can reveal differences in staining intensity between different Sertoli cells in the normal adult testis[159].

MAGE-57B antigen

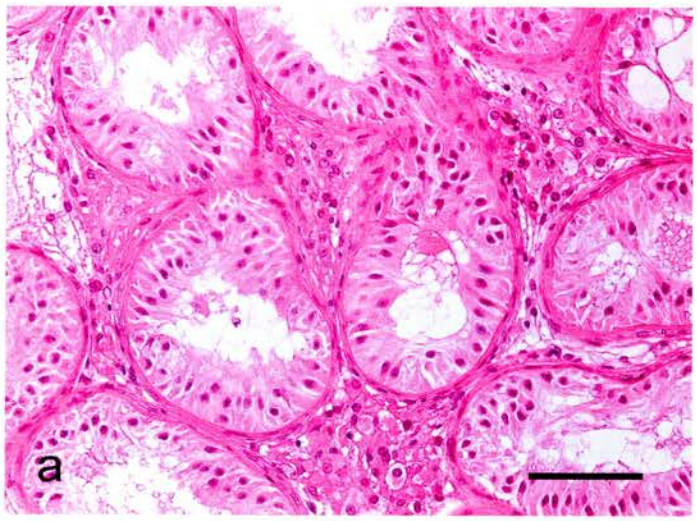
Immunoexpression of MAGE-57B was negative in all specimens, pre and post H-P-G axis suppression, in contrast to the abundant immunopositive germ cells seen in the healthy adult control (Figure 6.3(d)-(f)). This confirmed the absence of spermatogonia in the cancer patients.

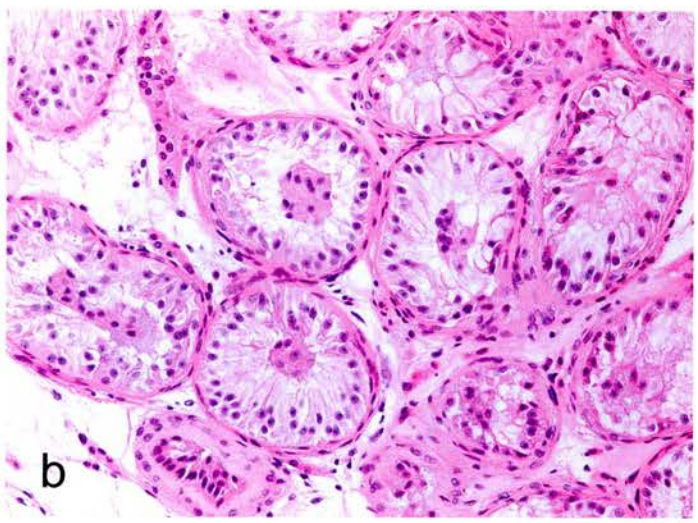
Inhibin α and βB subunits

Testicular tissue from all seven cancer survivors expressed inhibin α and -βB subunits in Sertoli and Leydig cells, and Leydig cells respectively and representative examples are illustrated in Figure 6.4(a) and (c) comparable to controls, panels (b) and (d).

3β-HSD and 17α-OH

Leydig cell immunoexpression of the steroidogenic enzymes, 3β-HSD and 17α-OH, in the cancer patients (Figure 6.4 (e) and (g) respectively) was comparable to expression in healthy adult controls (Figure 6.4 (f) and (h)).


a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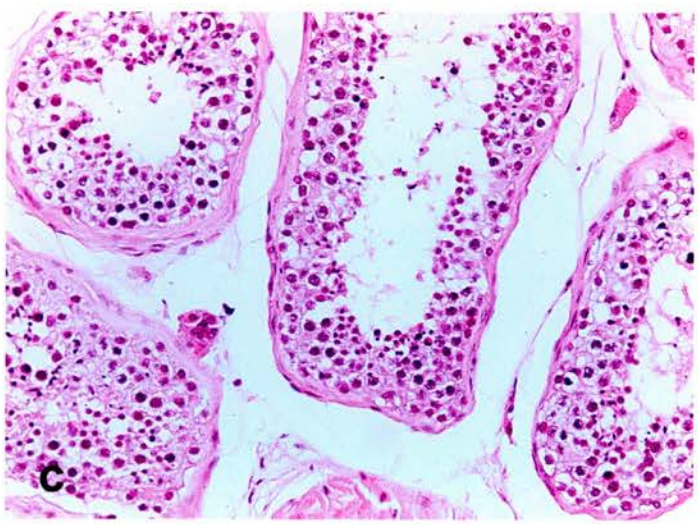

c

Figure 6.3.
Androgen receptor staining: Panels (a) and (b) show AR immunoexpression pre- and post- H-P-G axis suppression, respectively, and demonstrate that all cell nuclei within the seminiferous tubules are androgen receptor immunopositive (brown staining), thus excluding the presence of AR negative germ cells, as evident in the testis of a healthy adult man (c). Inset shows negative control in which the primary antibody was omitted. Scale bars denote 50 μm.

MAGE-57B staining: Panels (d) and (e) shows no evidence of expression of the MAGE-57B antigen (all cells stained blue), both pre-and post- H-P-G axis suppression. In contrast to the abundant germ cells immunopositive (brown staining) for MAGE-57B demonstrated in the healthy adult control (f). Insert shows negative control in which the primary antibody was omitted. Scale bars denote 50 μm.

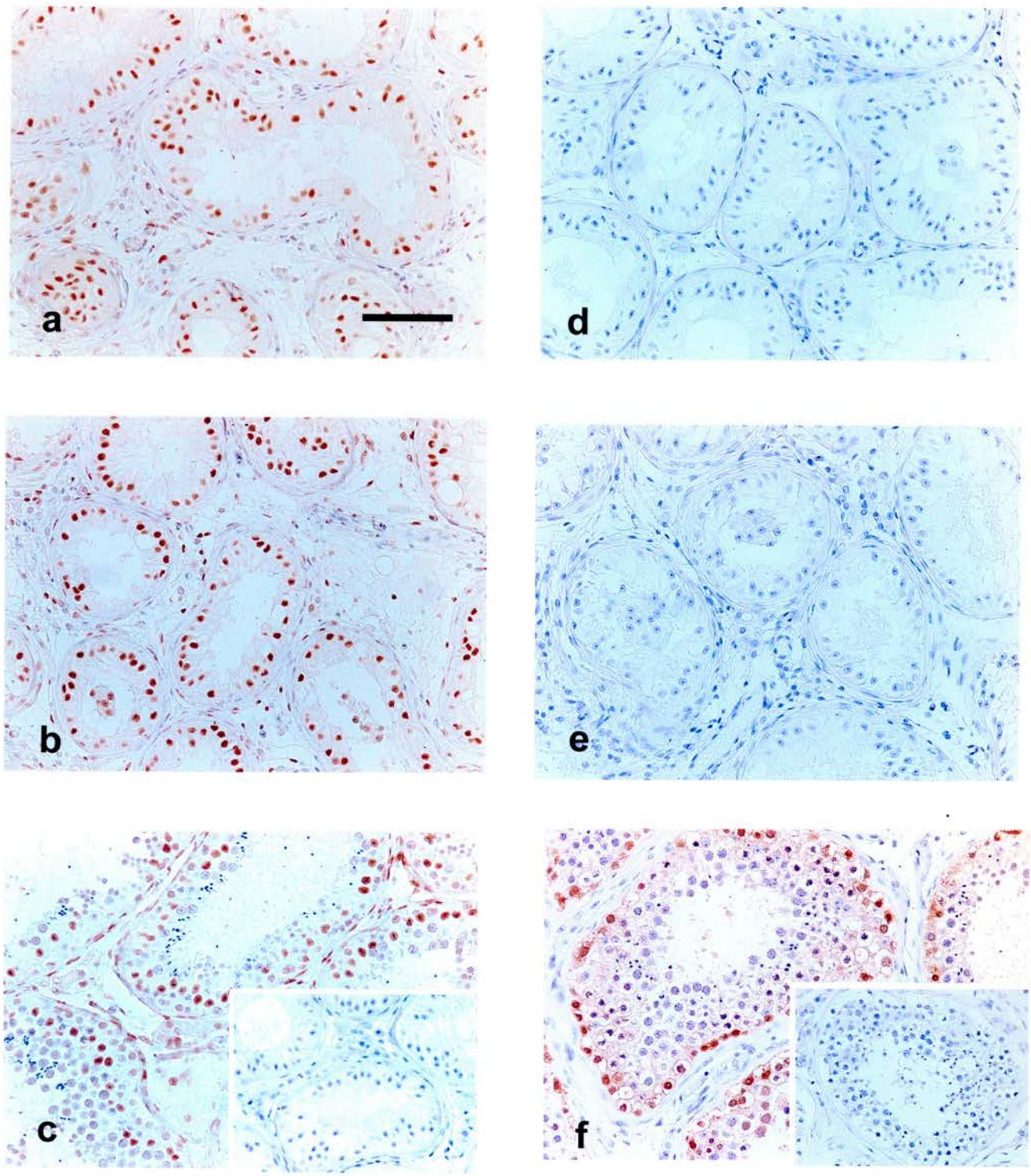

Androgen receptor
MAGE-57B
a
b
c
d
e
f

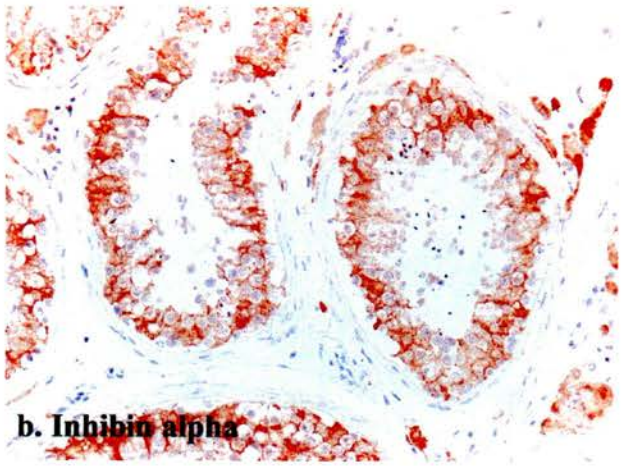
Figure 6.4.
Inhibin α and βB subunits. Panel (a) illustrates immunoexpression of inhibin α in Sertoli and Leydig cells of the testicular tissue from the cancer survivor, comparable to that of the healthy control sample shown in (b). Inhibin βB immunoexpression in Leydig cells is similar in the cancer survivor and in the healthy adult control, shown in panels (c) and (d) respectively.

3β-HSD and 17α-OH. Panels (e) and (g) illustrate immunoexpression of 3β-HSD and 17α-OH in Leydig cells in the cancer patients comparable to expression in the healthy adult controls, panels (f) and (h) respectively.

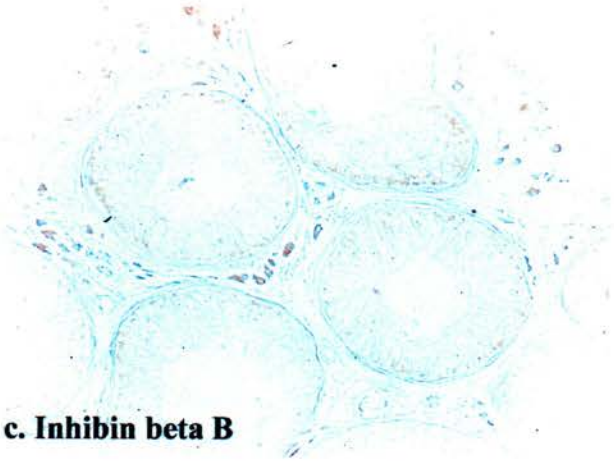
Patient

Control

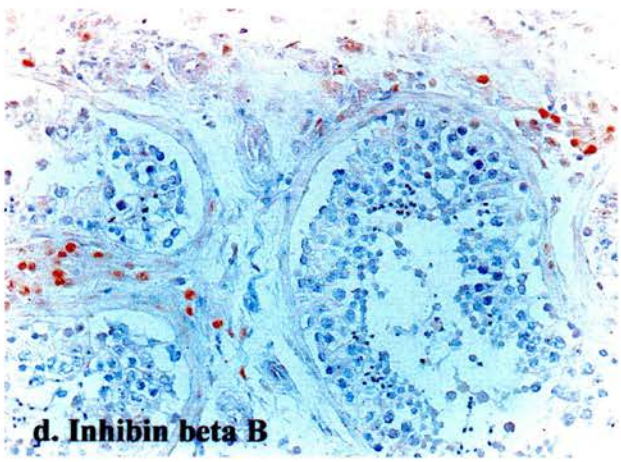
a. Inhibin alpha



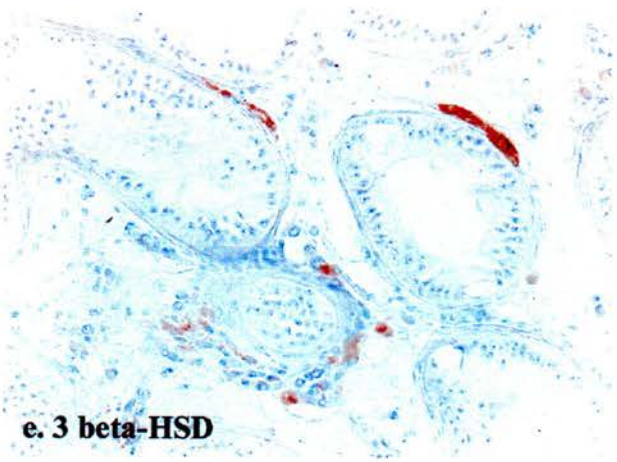
b. Inhibin alpha



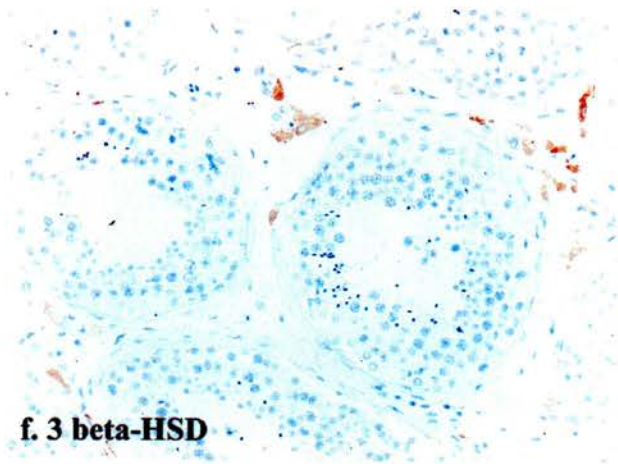
c. Inhibin beta B



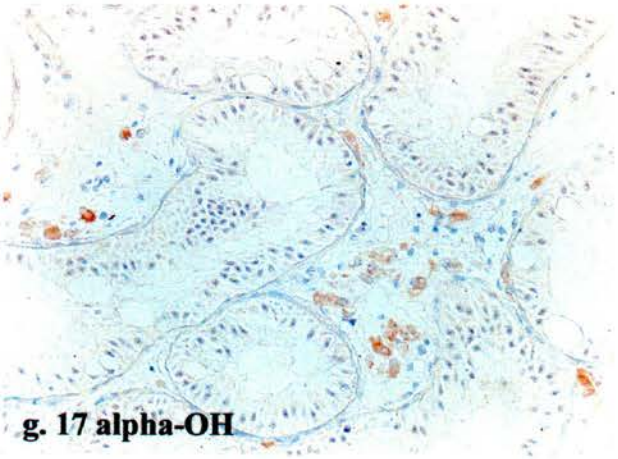
d. Inhibin beta B



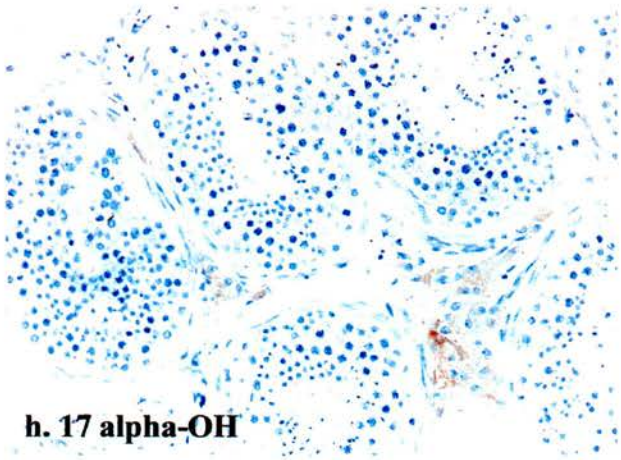
e. 3 beta-HSD



f. 3 beta-HSD



g. 17 alpha-OH



h. 17 alpha-OH

6.5. Discussion

This study demonstrates that suppression of the H-P-G axis for at least 3 months, in men rendered azoospermic by gonadotoxic chemotherapy or radiotherapy for childhood cancer did not result in restoration of spermatogenesis, assessed by both semen analysis and testicular biopsy. In rats, it has been shown that some germ cells survive some types of cytotoxic therapy and that the resulting azoospermia is a consequence of the inability of those spermatogonia that are present to proliferate and differentiate[40]. Suppression of the H-P-G axis facilitates recovery of spermatogenesis following such treatment, and it has been hypothesised that this is the result of a reduction in intratesticular testosterone concentrations[150]. This can be achieved by administration of steroid hormones, or GnRH agonists or antagonists with or without testosterone before, during or after chemotherapy or radiotherapy and such regimens have been demonstrated to enhance recovery of spermatogenesis in rats[144-149,160]. Application of this approach to humans makes the important assumption that the mechanism of cytotoxic chemotherapy- or radiotherapy-induced testicular damage is similar in both species, and it has been assumed that like the rat, men might retain a population of spermatogonial stem cells from which spermatogenesis could be regenerated. The mechanism and permanency of impaired spermatogenesis induced by some forms of chemotherapy/radiotherapy in the human may differ more substantially from the rat than was previously appreciated.

The success of hormonal treatment to aid recovery of spermatogenesis in rats subjected to chemotherapy is believed to be based on lowering intratesticular

testosterone levels. While the prepubertal testis is relatively quiescent there is a steady turnover of early germ cells, which undergo spontaneous degeneration before maturation is reached[35]. This relatively low activity, compared to the adult, does not protect the prepubertal testis from the deleterious impact of cytotoxic therapy, as the present data confirm. The slow turnover of germ cells and their subsequent degeneration in the prepubertal testis may be partly due to low levels of intratesticular testosterone, which is required to complete the end-stages of spermatogenesis[162]. The lack of protection afforded to the prepubertal testis, at a time when testosterone levels are low, would suggest additional environmental factors play a role in the successful recovery of spermatogenesis in rats and the vulnerability of the prepubertal human testis to cytotoxic therapy. In this regard, our studies with the marmoset, a primate surrogate for man, have demonstrated that activation of testicular cell function occurs well before puberty and is largely gonadotrophin-dependent, but that spermatogonial replication appears to be independent of gonadotrophin stimulation[33].

In one study in which cyclophosphamide was administered as immunosuppressive therapy for nephrotic syndrome in adult men, preservation of fertility was achieved via supraphysiological testosterone therapy[152]. Of fifteen men treated with cyclophosphamide, five received testosterone to suppress testicular function before and during the eight-month cycle of chemotherapy. All men were azoospermic or severely oligozoospermic within six months of commencing cyclophosphamide. Nine of the ten men who received cyclophosphamide alone remained azoospermic

six months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all five of the men who received testosterone therapy. High dose cyclophosphamide is known to be associated with impaired spermatogenesis, which is often temporary and it is probable that in this study the simultaneous administration of testosterone with cyclophosphamide provided some protection or hastened the recovery of spermatogenesis. It would be interesting to have long-term follow-up data on the ten patients who received cyclophosphamide only treatment to enable a direct comparison with the natural history of recovery of sperm production. In contrast, other studies have failed to show similar benefits in humans. For example, suppression of testicular function with a GnRH agonist, alone or in combination with testosterone during gonadotoxic chemotherapy treatment for lymphoma did not confer any protective benefit or enhance recovery of spermatogenesis[153,154]. A number of reasons may be considered for the lack of successful outcome in the aforementioned studies. The number of patients and controls studied was small and the cancer therapies variable, in contrast to monotherapy with cyclophosphamide for a non-malignant condition. Treatment regimens may not have been sufficiently gonadotoxic to cause sterility, so no recovery effect could be seen or, conversely, the agents were so gonadotoxic that permanent ablation of all germ cells was induced. Waxman and co-workers studied the protective effects of a GnRH agonist during the treatment of 20 men with cytotoxic chemotherapy for advanced Hodgkin's disease. Following administration of the GnRH agonist, standard GnRH testing demonstrated adequate suppression of LH, but not of FSH, throughout the chemotherapy treatment[154]. Follow-up

assessment of the men after a three year interval showed that all remained severely oligozoospermic[154]. In another study the effect of GnRH agonist administration during combination chemotherapy for advanced lymphoma was evaluated in six patients[153]. By six years post treatment, only one patient demonstrated any evidence of spermatogenesis. Similar unsuccessful results were obtained using GnRH analogues during cis-platinum chemotherapy for teratoma[155]. While the present study explored the delayed suppression aspect of this hypothesis, no human studies have combined pre-chemotherapy suppression with continued suppression for a significant length of time following chemotherapy.

A number of steroid hormone combinations have been used to suppress the H-P-G axis in rats and successfully restore spermatogenesis, including medroxyprogesterone acetate in combination with testosterone[40,162,163]. Low dose testosterone, medroxyprogesterone acetate or GnRH analogues alone have been shown to stimulate recovery of spermatogenesis in rats following sterilization with radiotherapy. However, the addition of testosterone to GnRH analogues may reduce the effectiveness of GnRH analogues[164]. The combination of testosterone with MPA may also have a reduced effect compared to either agent alone although this combination results in a profound reduction in intratesticular testosterone concentrations in men[165]. Although further study is warranted, the appropriate choice of hormone suppression will require careful consideration. Long-acting gonadotrophin analogues, such as goserelin have been shown to be ineffective at

suppressing FSH long-term in normal men, with recovery of FSH and resumption of spermatogenesis occurring within 2-3 months[166,167].

Inhibin B mediates non-steroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion[31,32,137,168]. Inhibin B secretion in the adult requires the presence of germ cells[31]. Inhibin B concentrations were barely detectable in the azoospermic patients, despite the preservation of Sertoli cells. Furthermore, the presence of the individual alpha and beta subunits were present, as identified by immunohistochemisry. This provides further evidence for an essential role of the germ cell-Sertoli cell interaction in the production of inhibin B and confirms the value of inhibin B as a non-invasive marker of spermatogenesis following cytotoxic therapy. Inhibin B was also undetectable in seminal plasma in most subjects, as previously found in men with azoospermia of other aetiologies[161].

All seven men had normal development of secondary sexual characteristics and reported normal potency. However, despite having testosterone concentrations within the normal range, hormone measurement demonstrated elevated LH levels, suggesting that there had been subtle damage to the Leydig cells. It was not possible to ascertain the degree of compensated Leydig cell function from immunohistochemical studies, as steroidogenic activity in cancer survivors appeared to be comparable to that of healthy controls, as assessed by intensity of immunoexpression of 17α-OH and 3β-HSD.

Although the gonadotoxic effect of chemotherapy depends upon dosage and drugs administered, and radiotherapy induced damage upon field of irradiation and dose received, it is difficult to reliably predict the extent of testicular damage and which azoospermic patients may show recovery of spermatogenesis. Our study population comprised an unselected group of seven men rendered azoospermic secondary to treatment for childhood cancer. Testicular biopsies from all seven patients demonstrated complete absence of spermatogonia, yet survival of stem cells is a prerequisite for endocrine restoration of spermatogenesis. It was felt to be unethical to exclude men from the trial on the basis of Sertoli cell only biopsy specimens for several reasons. Testicular volume in these men was markedly reduced and thus it was justified to take only a small piece of testicular tissue, to eliminate any impact a reduction in testicular tissue may have on Leydig cell numbers. Small islands of spermatogonia may be present but were not present in the biopsied tissue. Survival of germ cells following apparently sterilizing chemotherapy is evident from a number of studies. Temporary azoospermia and late recovery of spermatogenesis following chemotherapy have been reported, indicating the survival of stem cells,[45,50,57] although permanent azoospermia tends to follow procarbazine and alkylating agent-based regimens, typical of treatment for Hodgkin's disease[10,51,55]. Similar histological findings have been reported in other studies following treatment for Hodgkin's disease with procarbazine-based regimens[47,170]. With advances in assisted reproduction techniques, the development of testicular sperm extraction combined with intracytoplasmic sperm injection (TESE-ICSI) offers potential for paternity in these young men. Chan and co-workers report the use of TESE-ICSI to retrieve

sperm from men with long-standing azoospermia and achieve a pregnancy[171]. Seventeen men, median age (range), 37.4 (28-54) years had undergone sterilizing chemotherapy treatment 16.3 (6-34) years previously. Of the 17 men, 13 demonstrated Sertoli cell only on biopsy and the remaining four were described as having hypospermatogenesis. Using microdissection TESE techniques, sperm retrieval was achieved in 7 subjects, 3 (43%) of whom demonstrated Sertoli cell only on testicular histology and 4 (57%) hypospermatogenesis. The seven subjects underwent nine TESE combined with ICSI procedures resulting in a clinical pregnancy in 3 (33%) and a live birth in 2 (22%). These encouraging results suggest that microscopic visualization of the seminiferous tubules may enable identification of areas of continued spermatogenesis within the testis and sperm retrieval using microdissection techniques. This reiterates the importance of not excluding men from clinical trials of hormone restoration or from assisted reproduction techniques on the basis of a Sertoli-cell only biopsy.

We report for the first time the extent of testicular damage following cytotoxic chemotherapy and radiotherapy for childhood cancer and conclude that for these men recovery of spermatogenesis is unlikely. Although it is possible that small islands of germ cells exist in areas of tissues that were not biopsied, recovery of spermatogenesis is unlikely given the extent of destruction to the general tissue architecture. This does not exclude the possibility that earlier intervention and H-P-G axis suppression might have been beneficial. However, it seems more probable that H-P-G axis suppression to restore spermatogenesis may be more successful in

patients in whom the testicular insult is less severe and in whom there is some preservation of spermatogonial stem cells.

Chapter 7

Development of the prepubertal human testis: an immunohistochemical study

7. Development of the prepubertal human testis: an immunohistochemical study

7.1. Introduction

Infertility is a well recognised late sequela for a number of patients following treatment for childhood cancer. Consequently, there is tremendous impetus to develop strategies to preserve fertile potential in children undergoing sterilizing treatment for cancer.

The adult male reproductive system is highly susceptible to the toxic effects of radiation and chemotherapy. Toxic insults may directly destroy proliferating germ cells in the seminiferous epithelium, resulting in azoospermia, or, the effects may result indirectly from impairment of Leydig cell function (reduced production of sex steroids), or of Sertoli cell function. Sertoli cells are the key cell type regulating sperm production and provide physical and nutritional support for germ cells. The gonadotoxic effects of chemotherapy are well established, with the extent of cytotoxic damage being dependent on the agent used and the dose administered. The degree and permanency of testicular damage following radiotherapy depends on the field of treatment, total and fractionated dose of radiotherapy administered, and is inversely related to the age at time of treatment[7-15].

It is understandable that the adult testis is susceptible to damage from chemotherapy or radiotherapy because the process of sperm production involves the intense and

continuous proliferation of germ cells, including germ stem cells, and proliferating cells are targeted by either therapy. However, why radiation and chemotherapy should exert gonadotoxic effects in the 'quiescent', prepubertal testis is uncertain. This incongruity has been addressed via studies in a primate model, the marmoset monkey. These animals exhibit a similar developmental profile to the human male, in terms of having a neonatal period of testicular/hormonal activity, followed by a 'quiescent' period followed by puberty[33]. Using immunocytochemistry and cell quantification on sections of the testes of animals during the 'quiescent' period, these studies found that significant development/maturation of Sertoli, Leydig and germ cells occurs during this period (probably equivalent to age six to eight years in boys), which is long before the accepted time of onset of puberty in the marmoset[33]. From this, it was a working hypothesis that comparable cell division and/or maturation in the 'quiescent' testis of prepubertal boys is what renders the testis at this time susceptible to damage by cancer therapy. There is already indirect evidence that similar changes probably do occur in the 'quiescent' human testis as in the marmoset. For example, based on autopsy material, testis volume trebles and germ cell numbers increase steadily in the period from two years to prepuberty[35]. Other evidence has shown that gonadotrophin secretion in boys, especially nocturnal secretion, switches on many years prior to the time of clinically detectable puberty[29]. Although these studies in the marmoset provide a logical potential explanation for the susceptibility of the human prepubertal testis to damage by cancer therapy, evidence is lacking to confirm that changes to Leydig, Sertoli and germ cells, comparable to those that have been found in marmosets, also occur in the 'quiescent'

testis of prepubertal boys. If such changes can be demonstrated to occur, it would validate use of the marmoset as a model for the human in this instance and give encouragement to the possibility of using this primate model to develop a method of protecting spermatogenesis in boys undergoing cancer therapy prior to puberty.

The objective of this study was to establish the basis for why the testis in prepubertal boys is susceptible to the damaging effects of certain cancer therapies, such that fertility is compromised in adulthood. If it could be established that cell activity does occur in the 'quiescent' testis in boys and is comparable to changes shown in the marmoset, this will provide a rational basis for considering 'protective therapy' as well as validating use of the marmoset as a model for establishing safety and efficacy of such therapy. Testicular activity was assessed in testicular autopsy specimens from boys by evaluating maturational and/or proliferative changes in the main constituent cell types of the testis (Sertoli, Leydig and germ cells) using immunocytochemical techniques.

7.2. Methods

7.2.1. Tissue collection

7.2.1.1. Prepubertal testicular tissue

Prepubertal human testicular material was obtained from a tissue archive in the Pathology Department at the Royal Hospital for Sick Children, Edinburgh. This study was approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee. These specimens had been taken for diagnostic purposes at post mortem and would otherwise have been discarded. The specimens were supplied to us in an anonymised manner. Under these circumstances it is impossible to obtain the consent of these patients (or their parents), however, it was felt that the proposed studies using archived tissues would contribute in an important way towards our understanding of the developing testis. It is unacceptable to (surgically) sample testicular tissue from healthy boys during childhood in order to study testicular development and it was considered ethical to further our knowledge by studying the present supply of archive specimens, which would otherwise be discarded. This understanding of testicular development is a prerequisite to subsequent clinical studies in which a more informed assessment can be made of the potential for developing approaches that might protect sperm production in boys undergoing gonadotoxic cancer treatment.

The samples had been fixed in formalin and embedded in paraffin wax for storage at the time of collection and sections were cut for further investigations.

7.2.1.2. Control specimens

Adult human and marmoset testicular samples were obtained from an in-house tissue archive (gifted from the pathology department, University of Edinburgh and owned by Dr R. Sharpe and Dr P. Saunders); all had been fixed in Bouins.

7.2.1.3. Preparation of sections

Sections were cut to a depth of 5 microns, using a microtome (Leica, RM-2135) and slides were made using TESPA (3-Triethoxysilylpropylamine, Sigma-Aldrich) coated slides. The slides were dried in an oven at 37^0C overnight.

7.2.2. Light microscopy assessment

In order to assess the overall architecture of the tissue the sections were stained with haemotoxylin and eosin. Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed in water. The slides were immersed in haematoxylin for 5 minutes and then rinsed in water, followed by a brief immersion in alcohol (5-20 seconds) and a further washing in water. The slides were immersed in Scotts tap water for 30 seconds and rinsed in water. The sections were counter-stained with eosin, dehydrated in graded ethanols, cleared in xylene and cover-slipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK).

7.2.3. Immunohistochemical markers of testicular cell development

To assess the maturational and proliferative status of the Leydig, Sertoli and germ cells in the testes of boys of various ages, a variety of cell specific and non-cell specific markers were used.

Cell specific markers:

Sulphated glycoprotein-2 (SGP-2): this is a protein marker expressed in Sertoli cell cytoplasm and is a marker of Sertoli cell maturation/terminal differentiation in the rat. In the marmoset it has been shown to be immunoexpressed in Sertoli cells from mid childhood[172].

3β-hydroxysteroid dehydrogenase (3β-HSD): a steroidogenic enzyme involved in the biosynthesis of testosterone and is specific to Leydig cells or Leydig cell precursors[173]. In the marmoset 3β-HSD is immunoexpressed strongly during the neonatal period, tailing off during mid childhood and increasing again from puberty through to adulthood[172].

17α-hydroxylase (17α-OH): a steroidogenic enzyme expressed in Leydig cells[174].

C-kit: in the rat this is a plasma membrane marker for foetal germ cells (gonocytes). In the marmoset it is not expressed beyond a few weeks of age, as one would not expect to see gonocytes beyond this age[175].

MAGE-57B: the MAGE-57B antigen is expressed in gonocytes, equally strongly in spermatogonia and weakly in early spermatocytes in rats and humans[157].

Non-cell specific markers:

Androgen receptor (AR): is expressed in more than one cell type. In rat studies, AR is immunoexpressed in the Sertoli cell nuclei just before the onset of puberty, and also in peritubular cells and Leydig cells but never in germ cells[176,177]. Based upon previous studies in the marmoset, AR is a nuclear protein marker expressed only in Sertoli cells of seminiferous tubules of the prepubertal testis[33].

Inhibin α: this is one of the subunits of the dimeric glycoprotein, inhibin-B, which is secreted by the Sertoli cell and possibly by the Leydig cells in the testis, though various forms of the free α subunit may also be secreted[32,178]. It is a marker of Sertoli cell maturation[32,178].

Inhibin βB: this combines with inhibin α to form the glycoprotein heterodimer inhibin-B. It is secreted by Sertoli cells and is a marker of Sertoli cell maturation[31,32]. It is also expressed in germ cells and in Leydig cells in some species.

Proliferating cell nuclear antigen (PCNA): this is expressed widely during the cell cycle and is used as a marker of the rate of cell proliferation, or of 'non-quiescent' cells. Though this will target all dividing cells, it is anticipated that it will label

primarily germ cells at the ages relevant to the present investigation. PCNA has been used for similar purposes in many previous studies, including in the rat[179] marmoset[33,176]; rhesus monkey[179] and human[180].

Estrogen receptor α: Estrogens regulate germ cell function, mediated via high affinity estrogen receptors: estrogen receptor α (ERα), and estrogen receptor β (ERβ)[181,182]. Only the results of our studies of ERα are presented in this thesis, although studies are currently underway to explore immunoexpression of ERβ. ERα immunoexpression localises to efferent duct epithelial cells in marmosets and rats and may also target some interstitial cells, though only in the peripubertal period in the marmoset[33].

7.2.4. Antibodies

The first part of this study involved optimising conditions for human tissue for each of the immunohistochemical assays. Immunolocalization of 17α–OH and 3β-HSD utilized rabbit polyclonal antibodies against the human proteins (both gifted from Professor I Mason), both used at a dilution of 1:2000. C-kit was immunolocalized using a rabbit polyclonal antibody, used at a dilution of 1:50. Immunolocalization of inhibin βB used a monoclonal mouse antibody against rabbit βB (gift from Prof N Groome, Oxford Brookes University), at a dilution of 1:6000. MAGE-57B was immunolocalized using a mouse polyclonal antibody (Anti MAGE-A4 clone 57B; gift from G. Spagnoli), used at a dilution of 1:50. Immunolocalization of SGP-2

utilized a polyclonal antibody raised in sheep against human SGP-2 (gift from Dr J MacRae, Australia), used at a dilution of 1:3000. Immunolocalization of AR utilized a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) raised against an epitope at the N-terminus of human AR, at a dilution of 1:2000. Immunolocalization of inhibin-α utilized a monoclonal antibody (173/9K) described previously[182], and used at a dilution of 1:1000. Proliferating cell nuclear antigen (PCNA) was immunolocalized using a monoclonal antibody (PC10; Dako), and was used at a dilution of 1:500. ERα was immunolocalized utilizing a mouse monoclonal antibody (NCL-ER-6F11, Novocaster), used at a dilution of 1:20.

7.2.5. Immunohistochemistry

Unless otherwise stated, all incubations were performed at room temperature. Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed in water. A temperature-induced antigen retrieval step was required for androgen receptor, inhibin α- and β-B and 3β-HSD, ERα antigens. The sections were pressure cooked in 0.01M citrate buffer for androgen receptor, inhibin α- and 3β-HSD and ERα, or in 0.01M glycine buffer for inhibin β-B antigen, pH 6.0 for 5 minutes at full pressure, allowed to stand for 20 minutes, cooled in running tap water and washed twice in (5 minutes each wash) in Tris-buffered saline (TBS: 0.05 mol Tris-HCL, pH 7.4, 0.85% (w/v) NaCl). Endogenous peroxidase activity was then blocked by immersing sections in 3% (v/v) H_2O_2 in methanol (both from BDH Laboratory Supplies, Poole) for 30 minutes, followed by two 5 minute washes in TBS. Sections

were incubated for 30 minutes with the appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA: Sigma) to block non-specific binding sites. Normal swine serum (NSS) was used for androgen receptor, 3β-HSD and C-kit; normal goat serum (NGS) for 17α-OH and normal rabbit serum (NRS) for inhibin α- and β-B, MAGE-57B, ERα and SGP-2 (from Diagnostics Scotland, Carluke). Primary antibodies were added to the sections at the appropriate dilution, either in NSS-TBS-BSA, NRS-TBS-BSA, or NGS-TBS-BSA and incubated overnight at 4°C in a humidified chamber. The sections were washed twice in TBS and then incubated for 30 minutes with anti-rabbit or horseradish peroxidase-labelled polymer (EnVision: DAKO, Ely) for androgen receptor, 3β-HSD, 17α-OH and C-kit, or anti-mouse for inhibin α- and β-B, MAGE-57B and ERα, and anti-sheep for SGP-2. Sections were washed twice (5 minutes each) in TBS and immunostaining was developed using Liquid DAB (DAKO) until staining was optimal, when the reaction was stopped by immersing sections in distilled water. The sections were counter-stained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody.

Immunostained sections were examined and intensity was graded as '+': weak staining; '++': moderate staining; '+++': intense staining. Relative numbers of cells in a particular location staining positive was graded '1 to 5', with '1' representing

only a few cells staining positive, to '5' reflecting all cells staining positive. The sections were photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, UK) fitted with a digital camera (Kodak DCS330: Eastman Kodak, Rochester, NY, USA). Captured images were stored on a computer (G4; Apple Computer Inc., Cupertino, CA) and compiled using Photoshop 5.0 before printing using an Epson Stylus 870 colour printer (Seiko Epson Corp., Nagano, Japan).

7.3. Results

The objective of this study was to obtain post mortem testicular tissue samples from preterm infants (from 23 weeks gestation onwards) through to puberty. It had been hoped to identify at least six tissue samples from each age bracket in order to ensure good representation and to enable conclusions to be reached about testicular development during each time period. Age brackets were assigned as follows: 23-30 weeks gestation (n=13); 31-40 weeks gestation (n=22); 0-1 month (n=11); 1-6 months (n=26); 7-12 months (n=6); 1-5 years (n=10); 6-10 years (n=7); 11-15 years (n=1). Post mortem testicular tissue was abundantly available from premature babies up to infants aged 12 months, but thereafter samples were fewer in numbers. Unfortunately, due to the availability of testicular tissue it was not possible to include as many tissue specimens from older patients as had been anticipated. As the study continues it is hoped that the goal of six or more samples in each age group will ultimately be achieved. In addition, at the time of collection of the original testicular tissue the whole testes were taken and fixed in formalin for a variable time before embedding in paraffin wax. The testes had not been weighed at the time of collection, thus it was not possible to make an assessment about the volume of the testes at different ages and therefore not possible to derive absolute numbers of particular cell types per testis. Furthermore, as the precise fixation procedures were not specified and may have been more optimal for some specimens than for others (e.g. due to testis size) the quality of the tissue undoubtedly varied in quality between specimens. Differences in timing between death and tissue fixation was also likely to

have varied considerably and this may have differentially affected the preservation of certain antigens. Such factors had to be taken into account in the studies described.

The immunohistochemical techniques had been established for rats and marmoset tissues and a significant period of time was spent optimising the conditions for human tissues. A compounding factor for the lack of success when applying the procedures as determined for the marmoset, was that the marmoset testicular tissue had been carefully dissected free of connective tissue, fixed in Bouin's fluid for a defined period of time, after which the testis was dissected from the epididymis and weighed, before processing the fixed testis overnight and embedding in paraffin.

7.3.1. Light microscopy

During infancy and early childhood, seminiferous cords were present with no lumens. Sertoli cells were abundant with a few germ cells and the occasional binucleated spermatogonium (Figure 7.1(a)-(h)). Seminiferous tubule lumens were only evident in tissue from the 12yr old patient, which was the only specimen available for the age bracket 11-15 years (Figure 7.1(h)). Germ cells were more abundant in the older patients with some evidence of differentiation to spermatocytes (Figure 7.1(h)). A healthy adult testis is shown for comparison (Figure 7.1(i)).

An interesting finding from these studies was the relatively large size of the efferent ducts in relation to the size of the testis during third trimester development and infancy (Figure 7.1(a)). This is a new finding of uncertain significance, perhaps

significant of a larger, as yet undefined role of the epididymis, and warrants further assessment.

7.3.2. Sertoli cell development (Table 7.1)

Evaluation of Sertoli cell development and maturation was made using immunoexpression of the protein markers AR, SGP-2, inhibin α and βB. Nuclear expression of AR in certain testicular cell types was evident from 23 weeks gestation to early puberty, as shown in figure 8.2. However, AR immunoexpression in Sertoli cells was negative until the onset of puberty (Figure 7.2(a)-(h)). This is in contrast to the earlier appearance of AR from 'mid childhood' in the marmoset[33]. A significant degree of background staining was evident in figure 7.2(c), which reiterated the difficulties associated with optimising the staining techniques. SGP-2 immunoexpression was not detectable in any of our specimens from 23 weeks gestation through to 12 years of age. SGP-2 is a marker of Sertoli cell maturation in the rat and marmoset and these findings reflect the immature state of development in these subjects, although immunoexpression of SGP-2 was expected in the older subjects from 9-12 years, if the marmoset model is a true reflection of human prepubertal testicular development. Inhibin-α was abundantly expressed in Sertoli cells of all ages and appeared to decrease in intensity with increasing age (Figure 7.3(a)-(i)). Inhibin-α was expressed most intensely in Sertoli cells in the preterm and neonatal testes (Figure 7.3(a)-(c)). During mid childhood, inhibin-α was only immunoexpressed at low intensity (Figure 7.3(f) and (g)). Inhibin-βB was

immunoexpressed in Sertoli cells in the seminiferous cords during 30-40 weeks of gestation and was then absent during infancy and early childhood (Figure 7.4(b)-(f)). Immunoexpression of inhibin βB was detected in Sertoli cells during mid childhood, age 6-10 years, and weakly present in the peripubertal testis (Figure 7.4(g) and (h)). Inhibin βB was abundantly immunoexpressed in germ cell, Sertoli cells and Leydig cells of a healthy adult control (Figure 7.4(i)).

7.3.3. Leydig cell development (Table 7.1)

Expression of 3β-HSD was confined to the cytoplasm of Leydig cells. In the prepubertal human testis, 3β-HSD was immunoexpressed intensely and abundantly during weeks 23-30 of gestation (Figure 7.5a). During the last 10 weeks of foetal life and infancy immunoexpression remained intense but fewer cells were immunopositive. There was no 3β-HSD immunoexpression during childhood (Figure 7.5(e)-(h)). A healthy adult testis illustrates intense 3β-HSD immunoexpression in the Leydig cells (Figure 7.5(i)). In the marmoset, immunoexpression of 3β-HSD was relatively intense during the neonatal period and then declined during early to mid childhood (20-35 weeks) before increasing markedly at puberty through to adulthood[33].

Inhibin α immunoexpression was weakly positive throughout preterm life until six months of age in the human, and subsequently became negative during childhood, with very weak expression observed in children of pubertal age (Figure 7.3(a)-(h)).

Inhibin α immunoexpression in adult human Leydig cells is shown for comparison (Figure 7.3(i)). In marmosets it was also possible to demonstrate an increase in Leydig cell number and functional capacity of the cells from mid childhood (35 weeks), through puberty to adulthood[33]. Based on Leydig cell nuclear volume it was shown that the number of Leydig cells increased ≅5-fold, whereas cytoplasmic volume per testis increased by up to 10-fold between mid childhood and adulthood in the marmoset[33]. This sort of evaluation was not possible in our studies as the testicular volumes were not available.

AR was also immunoexpressed in interstitial cells in the human testis, being maximal during foetal life and the neonatal period (Figure 7.2(a)-(c)) and subsequently decreasing during infancy (Figure 7.2(d) and (e)). AR immunoexpression in peritubular cells remained weakly positive throughout childhood but Leydig cells remained immunonegative for AR until early puberty in all human specimens studied (Figure 7.2(e)-(h)).

17α-OH, another cytoplasmic marker of steroidogenic activity in Leydig cells was intensely expressed in the very preterm infants, born between 23 and 30 weeks of gestation and was less intensely and abundantly expressed during 30 to 40 weeks of gestation (Figure 7.6(a) and (b)). Immunoexpression of 17α-OH was absent throughout childhood and remained undetectable in the peripubertal testis (Figure 7.6(c)-(h)), in contrast to positive immunoexpression in a healthy adult control

(Figure 7.6(i)). 17α-OH immunoexpression appears to switch off a little earlier than 3β-HSD, but it is uncertain whether this discrepancy reflects a true pattern of steroidogenic activity or is indicative of differences in antigen preservation/detection/sensitivity. It was not possible to compare these findings with the marmoset during this study.

Inhibin βB immunoexpression was detected in small numbers of Leydig cells in some of the samples (Figure 7.3.). Expression was most intense in the preterm infants, becoming weakly positive to undetectable by the end of the first year of life and becoming weakly detectable again peripubertally.

ER-α was not immunoexpressed in the Leydig cells in any of our human specimens studied. ER-α localises to Leydig cells in the rat and may also target some interstitial cells, though only in the peripubertal period in the marmoset[33]. In addition, ER-α immunoexpression localises to efferent duct epithelial cells in marmosets and rats Positive immunoexpression of ER-α was evident and in the efferent duct epithelial cells during foetal life and throughout childhood (Figure 7.7(a)-(g)). It was not possible to stain for ER-α in the peripubertal testis, as the tissue specimen obtained did not include efferent ducts. ER-α expression was not detected in the rete testis or epididymis.

During studies with the germ cell marker c-kit, cytoplasmic immunoexpression of c-kit in the interstitial cells was also detected (Figure 7.8). C-kit was immunoexpressed intensely in interstitial cell cytoplasm during 23-30 weeks of gestation (Figure 7.8(a)). There were small numbers of cells stained weakly positive during infancy (Figure 7.8(d)). Interestingly, in the foetal samples c-kit immunoexpression in interstitial cells was evident in the cytoplasm and around the perinuclear membrane in some of the cells (Figure 7.8(a)).

7.3.4. Germ cell development (Table 7.1)

Identification and subsequent quantification of germ cells during testicular development is hampered by the lack of type-specific germ cell markers. In this study we used c-kit and MAGE-57B as markers to identify germ cells. C-kit is a germ cell plasma membrane marker, which is specific for gonocytes and early spermatocytes in the developing rat. In our studies, c-kit expression was present in gonocytes during foetal life, most intensely during 30-40 weeks of gestation (Figure 7.8(a) and (b)). Less intense staining was detected up to 6 months of age (Figure 7.8(c) and (d)) but was absent thereafter (Figure 7.8(e)-(h)). In the marmoset, c-kit is not expressed beyond the first few days of life, in term monkeys, and has not been studied in premature infants.

Immunoexpression of MAGE-57B was detected in germ cells from the very preterm infants through to peripubertal subjects (Figure 7.9(a)-(h)). Intensity of staining was relatively constant at all ages but there appeared to be more abundant staining during

foetal life (Figure 7.9(a) and (b)), with fewer cells staining during infancy and early childhood (Figure 7.9(c)-(e)) and increasing in frequency again from mid childhood onwards (Figure 7.9(f)-(h)). It was not possible to make an assessment of the differential expression within the different stages of germ cell development. The MAGE-57B antigen is expressed in early germ cells, strongly in spermatogonia and weakly in early spermatocytes in rats, and studies are currently underway to explore its expression in the marmoset.

7.3.5. Cell proliferation (Table 7.1)

Immunohistochemistry studies using the nuclear marker PCNA were unsuccessful in our specimens. PCNA immunoexpression occurred in at most only one or two cells per high-powered field and appeared to immunostain Sertoli cells rather than germ cells. An example is shown in figure 7.10(a). While this finding may simply indicate that germ cells were not proliferating at the ages studied, the consistent lack of germ cell expression of PCNA throughout the range of ages would suggest that this antigen was not detectable in these tissues for technical reasons, such as poor preservation of the antigen. A testis from a prepubertal marmoset with widely expressed PCNA is shown for comparison (Figure 7.10(b)).

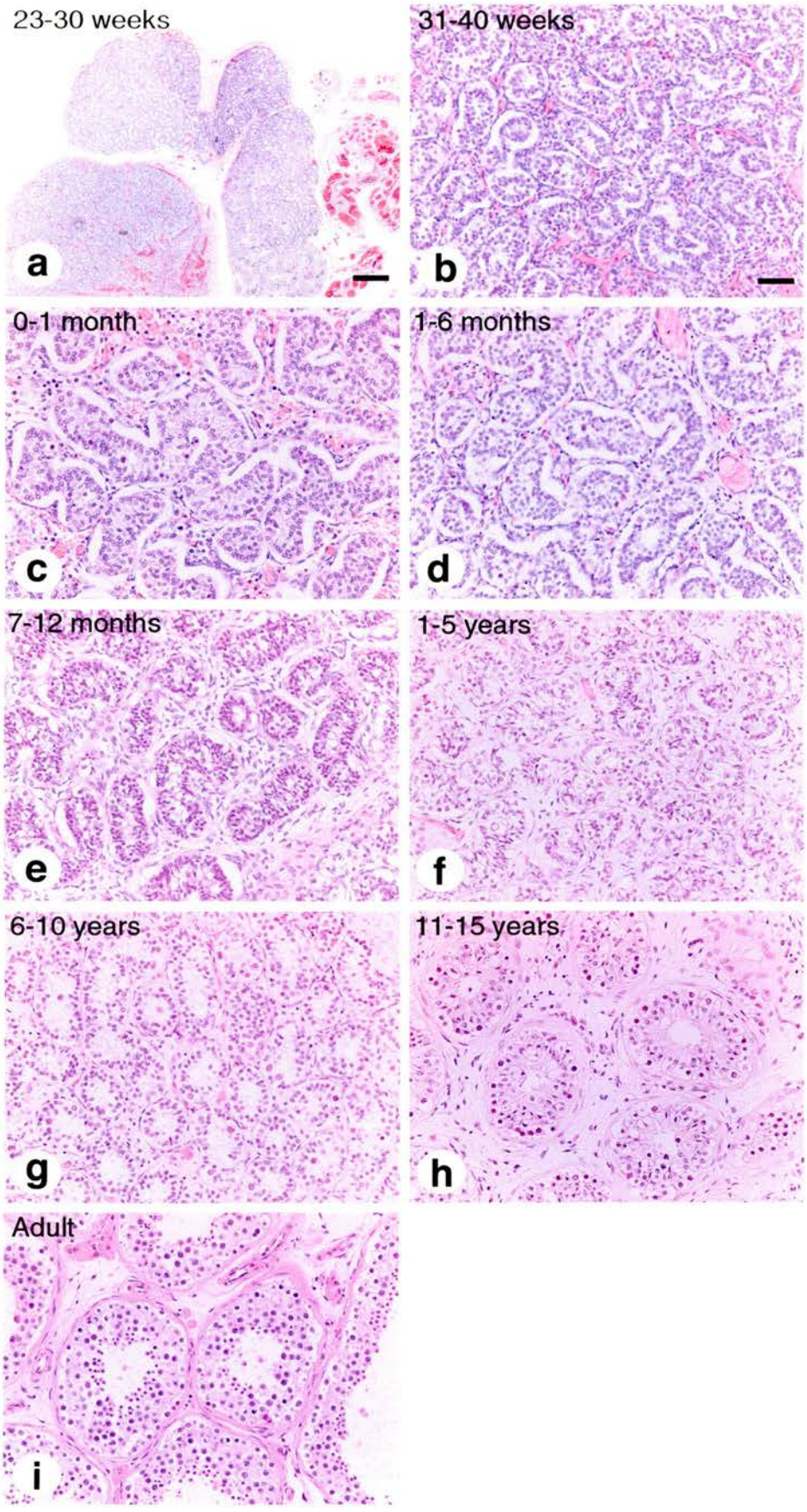
A summary of immunoexpression of the various markers used to evaluate maturational and / or proliferative changes in the main constituent cell types of the testis is shown in Table 7.1. Positive immunoexpression of a given marker is indicated by '+' for each age bracket and absence of a marker is indicated by '-'.

Table 7.1. Summary of immunoexpression of markers of development of the prepubertal testis.

Age	Sertoli cell markers				Leydig cell markers						Germ cell markers			Efferent ducts
	AR	Inhibin α	Inhibin βB	SGP-2	3β-HSD	17α-OH	Inhibin α	Inhibin βB	ER-α	C-kit	MAGE-57B	PCNA	C-kit	ER-α
23-40 weeks gestation	-	+	-	-	+	+	+	+	-	+	+	-	+	+
31-40 weeks gestation	-	+	+	-	+	+	+	+	-	-	+	-	+	+
birth – 1 month	-	+	-	-	+	-	+	-	-	-	+	-	+	+
1-6 months	-	+	-	-	+	-	-	-	-	+	+	-	+	+
7-12 months	-	+	-	-	-	-	-	-	-	-	+	-	-	+
1-5 years	-	+	-	-	-	-	-	-	-	-	+	-	-	+
6-10 years	-	+	+	-	-	-	-	-	-	-	+	-	-	+
11-15 years	-	+	+	+	-	-	+	-	-	-	+	-	-	N/A

+ : immunoexpression of the marker detected in testicular tissue from the defined age bracket; - : the marker was not detected in testicular tissue from the defined age bracket

Figure 7.1. Haematoxylin and eosin staining: Panels (a) to (h) demonstrate testis morphology from 23 weeks gestation through childhood to early puberty. Seminiferous cords are evident with abundant Sertoli cells and only a few germ cells during foetal life and early childhood. Seminiferous tubules with luminal development are demonstrated in the peripubertal testis (h). Panel (a) also illustrates the relatively large size of the efferent ducts in relation to the size of the testis, which is seen in the third trimester of foetal development and infancy. Scale bar denotes 50 μm.


23-30 weeks
a
31-40 weeks
b
0-1 month
c
1-6 months
d
7-12 months
e
1-5 years
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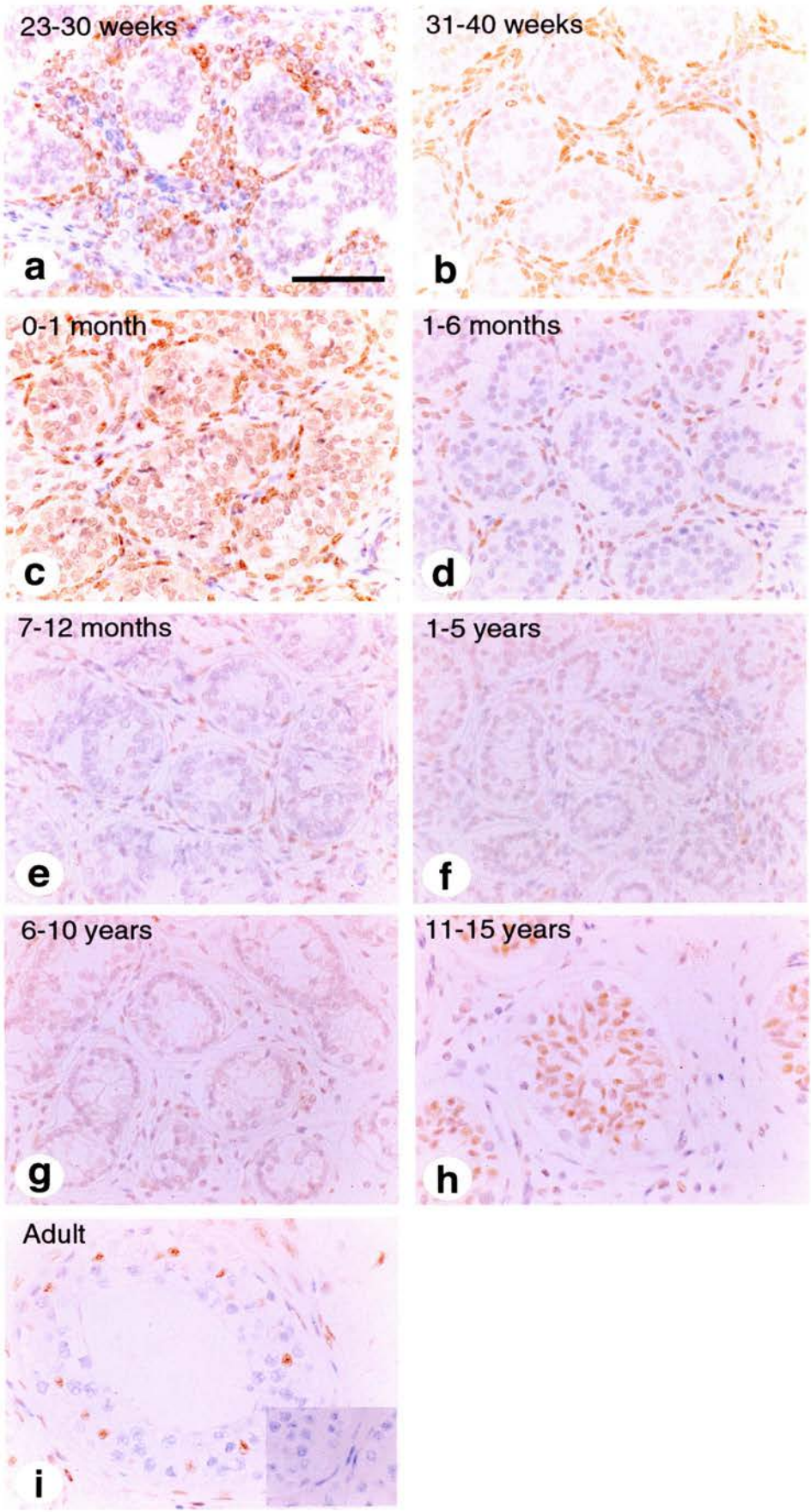
6-10 years
g
11-15 years
h
Adult
i

Figure 7.2. Androgen receptor immunoexpression: Panels (a) to (h) show AR immunoexpression during foetal life and childhood. AR expression in Sertoli cells was negative during foetal life and childhood, (a) to (g), until the onset of puberty (h). A significant degree of background staining was seen in panel (c). AR immunoexpression was present in interstitial cells, with a maximum intensity during weeks 23-30 of foetal gestation and decreasing thereafter. Peritubular cells immunoexpressed AR during infancy and early childhood, (a) to (g), but immunoexpression was not detectable in peritubular cells during early puberty (h). A healthy adult testis is shown for comparison (i). Inset shows the negative control in which the primary antibody was omitted. Scale bar denotes 50 μm.


23-30 weeks
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0-1 month
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7-12 months
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1-5 years
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6-10 years
g
11-15 years
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Adult
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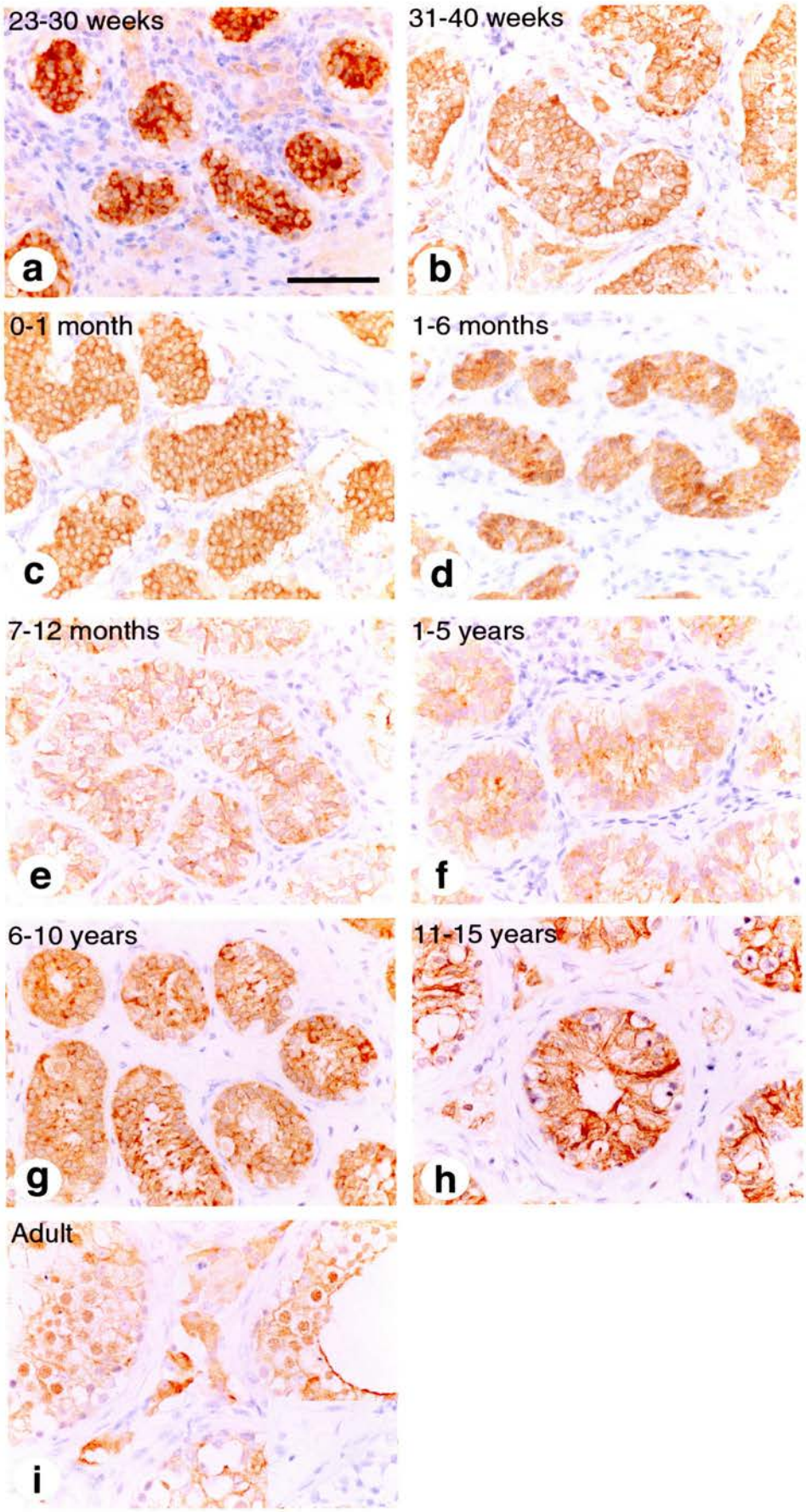
Figure 7.3. Inhibin α immunoexpression: Panels (a) to (h) show inhibin α immunoexpression in Sertoli cells. Inhibin α was abundantly expressed in Sertoli cells at all ages and appeared to decrease in intensity during early childhood, (e) and (f). Some immunostaining for inhibin α was also evident in interstitial cells at some of the ages, panels (a), (b), (c) and (h). A healthy adult control is shown for comparison (i) and the inset shows the negative control from which the antibody was omitted. Scale bar denotes 50 μm.


23-30 weeks
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7-12 months
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1-5 years
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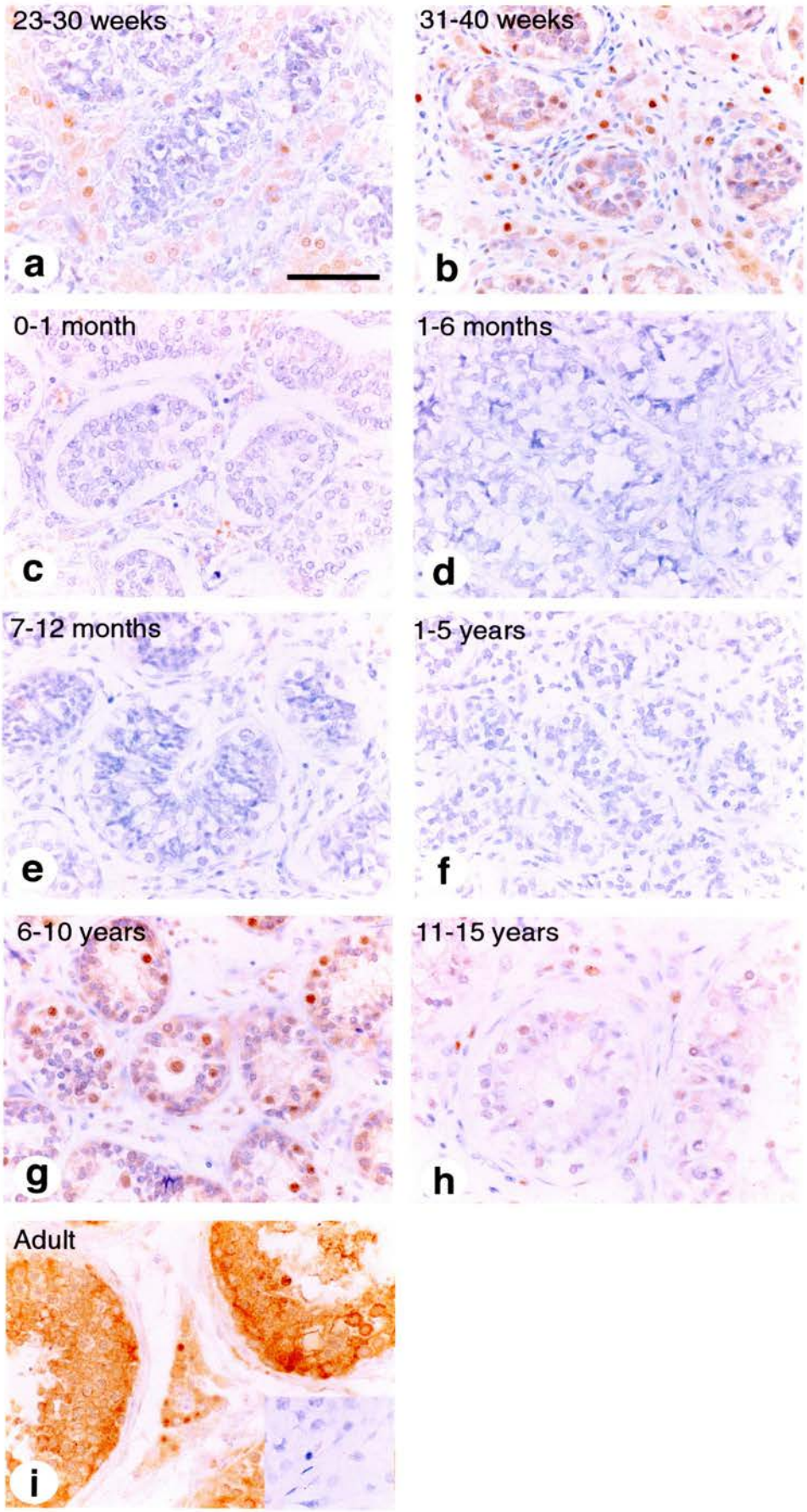
6-10 years
g
11-15 years
h
Adult
i

Figure 7.4. Inhibin βB immunoexpression: Inhibin βB was immunoexpressed in Sertoli cells at low intensity in the seminiferous cords during 30-40 weeks gestation (b) but immunoexpression was not detectable during early childhood, panels (c) to (f). Inhibin βB was immunoexpressed in both germ cells and Sertoli cells during mid puberty (g) and was weakly expressed in the peripubertal testis (h). Inhibin βB was immunoexpressed in interstitial cells during foetal life, panels (a) and (b). For comparison, a healthy adult testis is shown with abundant immunoexpression of inhibin βB in Sertoli cells, germ cells and interstitial cells. Inset shows negative control. Scale bar denotes 50 μm.


23-30 weeks
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0-1 month
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7-12 months
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1-5 years
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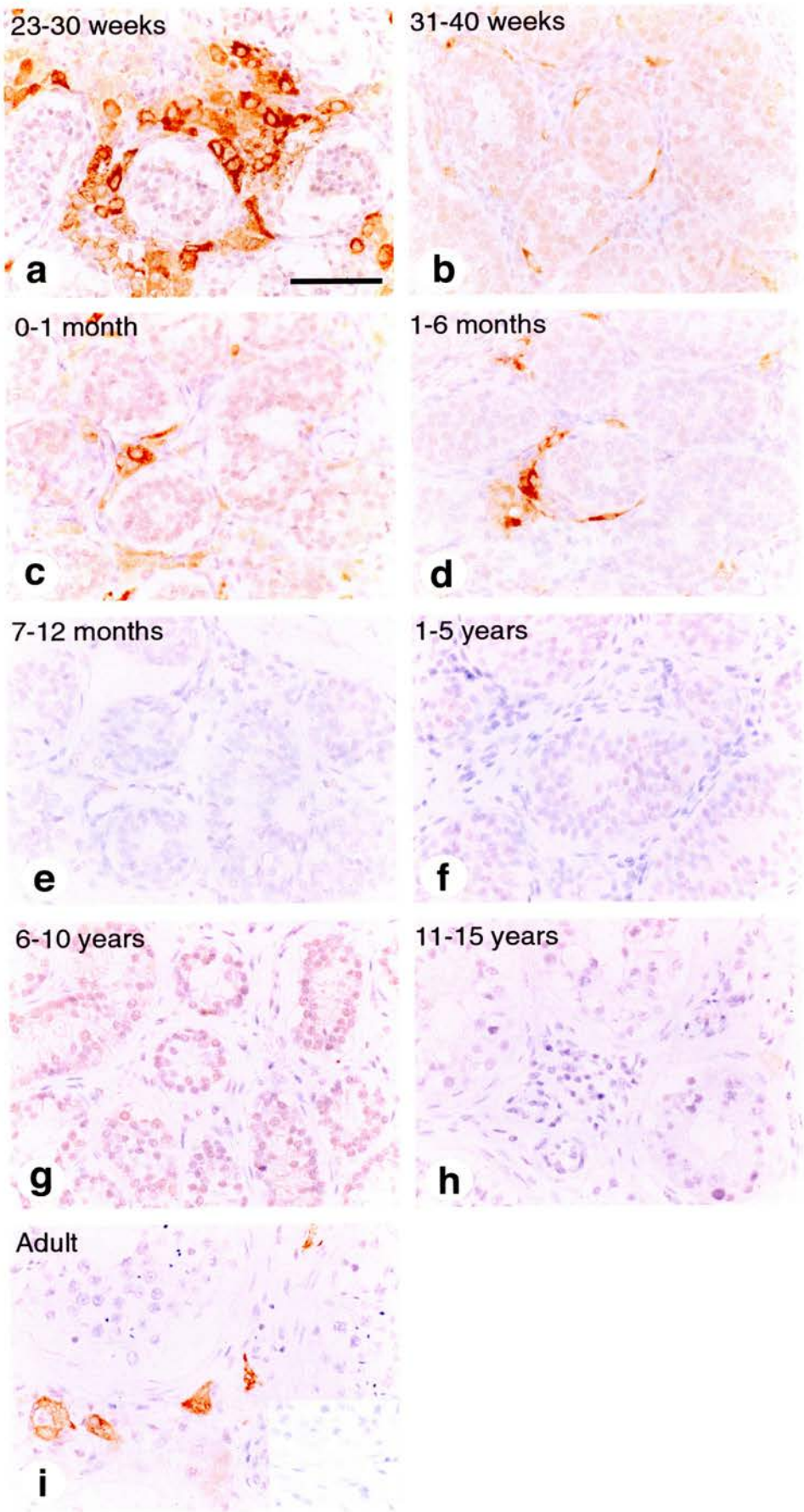
6-10 years
g
11-15 years
h
Adult
i

Figure 7.5. 3β–HSD immunoexpression: Panel (a) shows intense and abundant immunoexpression 3β–HSD during 23-30 weeks of gestation. Intense but less abundant 3β–HSD staining was evident between 30 weeks of gestation and 6 months of age, panels (b) to (d). 3β–HSD immunoexpression was not detectable from age 6 months onwards up to puberty, panels (e) to (h). A healthy adult control expressing 3β–HSD in Leydig cells is shown for comparison with negative inset (i). Scale bar denotes 50 μm.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7-12 months
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1-5 years
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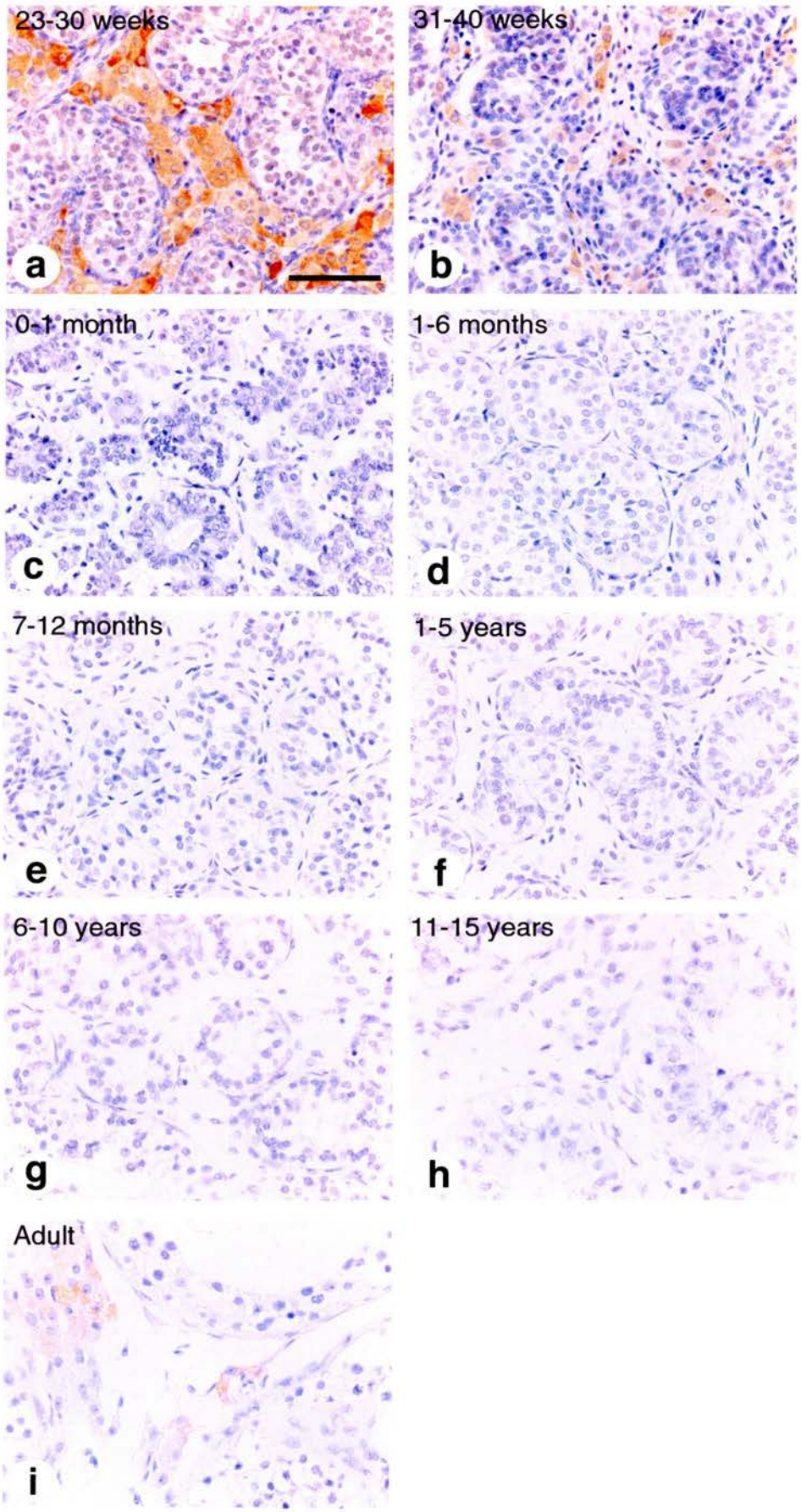
6-10 years
g
11-15 years
h
Adult
i

Figure 7.6. 17α-OH immunoexpression: Panels (a) and (b) show intense staining and abundant immunoexpression of 17α-OH during 23-30 weeks of gestation with less intense immunoexpression during 30-40 weeks of gestation. Thereafter 17α-OH immunoexpression was not detectable, even in the prepubertal (h) samples. A healthy adult exhibiting weak immunoexpression of 17α-OH in Leydig cells is shown for comparison with negative inset (i). Scale bar denotes 50 μm.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11-15 years
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Adult
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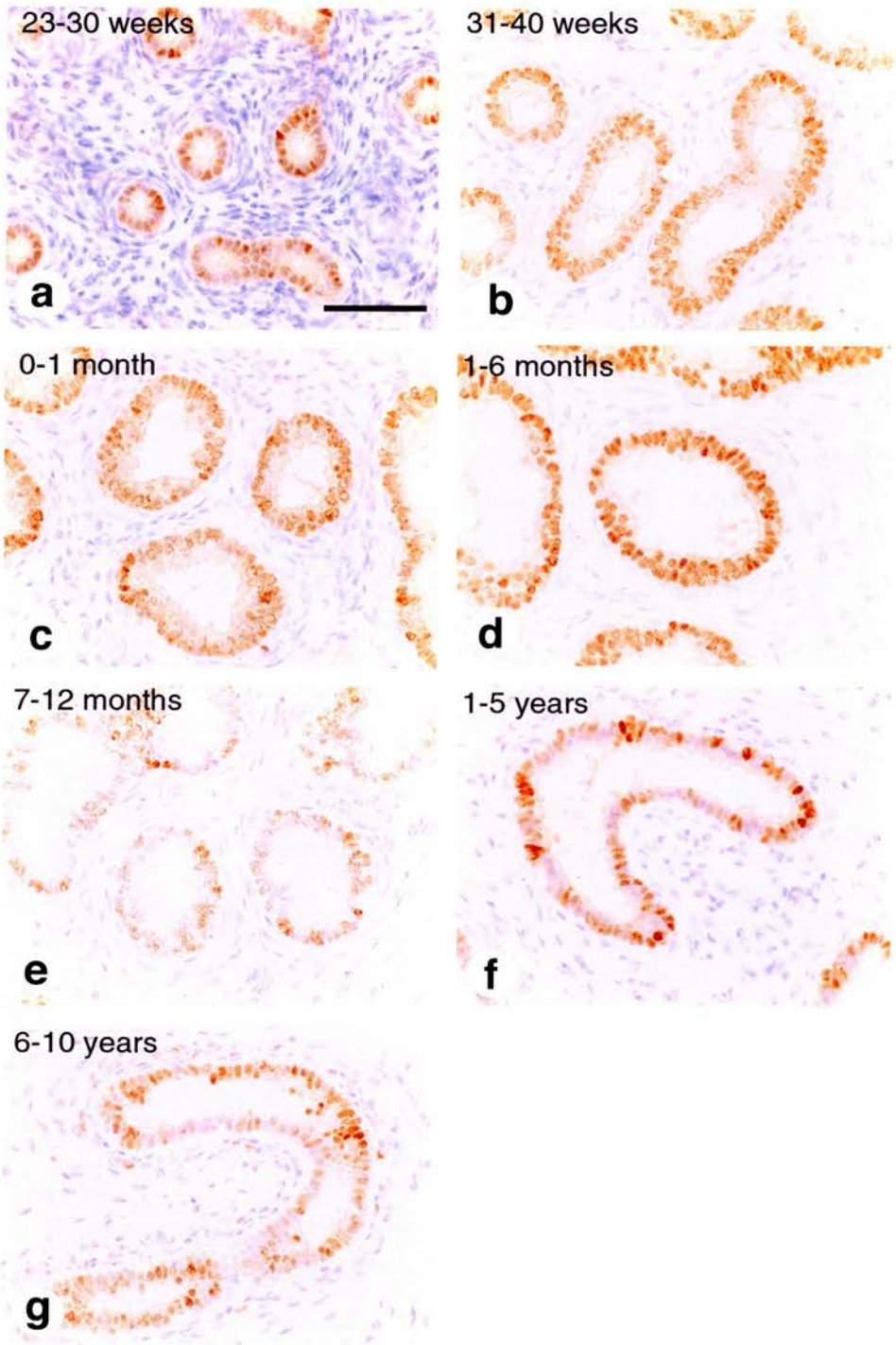
Figure 7.7. ER-α immunoexpression: Panels (a) to (d) show intense immunoexpression of ER-α in the efferent duct epithelial cells from 23 weeks gestation until 6 months of age and thereafter, where immunoexpression was less intense. There was a notable increase in size of the ducts with increasing age, panels (a) to (g). Scale bar denotes 50 μm.


23-30 weeks
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1-5 years
f
6-10 years
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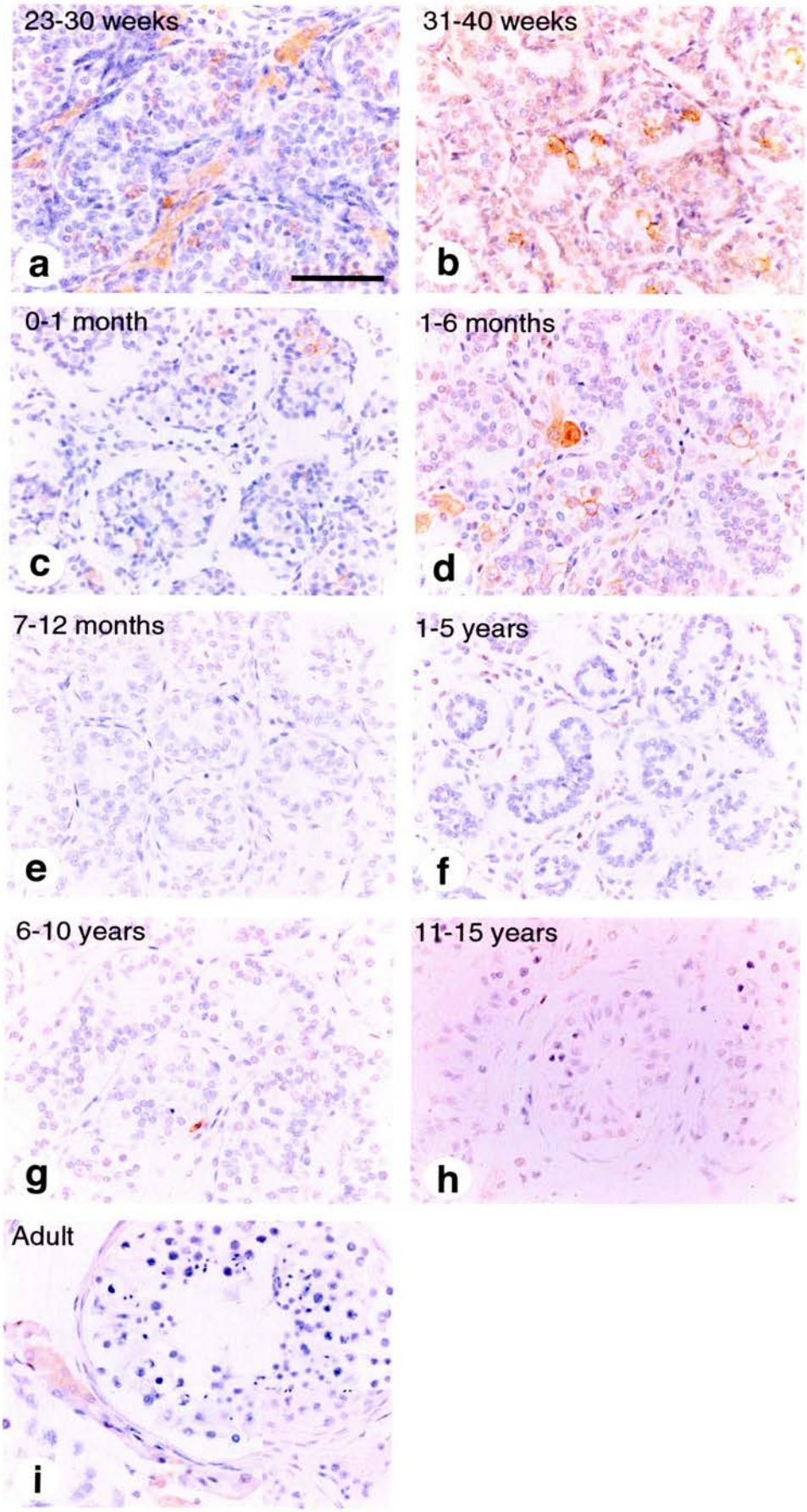
Figure 7.8. c-kit immunoexpression: Panels (b), (c) and (d) show immunoexpression of c-kit on the plasma membrane of the very early germ cells, or gonocytes, from 23 weeks of gestation until 6 months of age. Immunoexpression of c-kit on the plasma membrane of the gonocytes is most intense in the specimens aged 30-40 weeks of gestation and 1-6 months of age. Immunoexpression of c-kit is not detected on the plasma membrane of germ cells from 6 months of age onwards, panels (e) to (h), reflecting absence of gonocytes from this age onwards. Cytoplasmic expression of c-kit is evident in Leydig cells at certain ages, panel (a) and (d), 23-30 weeks of gestation and 1-6 months of age respectively. A healthy adult is shown for comparison, with no immunoexpression of c-kit detected, and negative inset (i). Scale bar denotes 50 μm.


23-30 weeks
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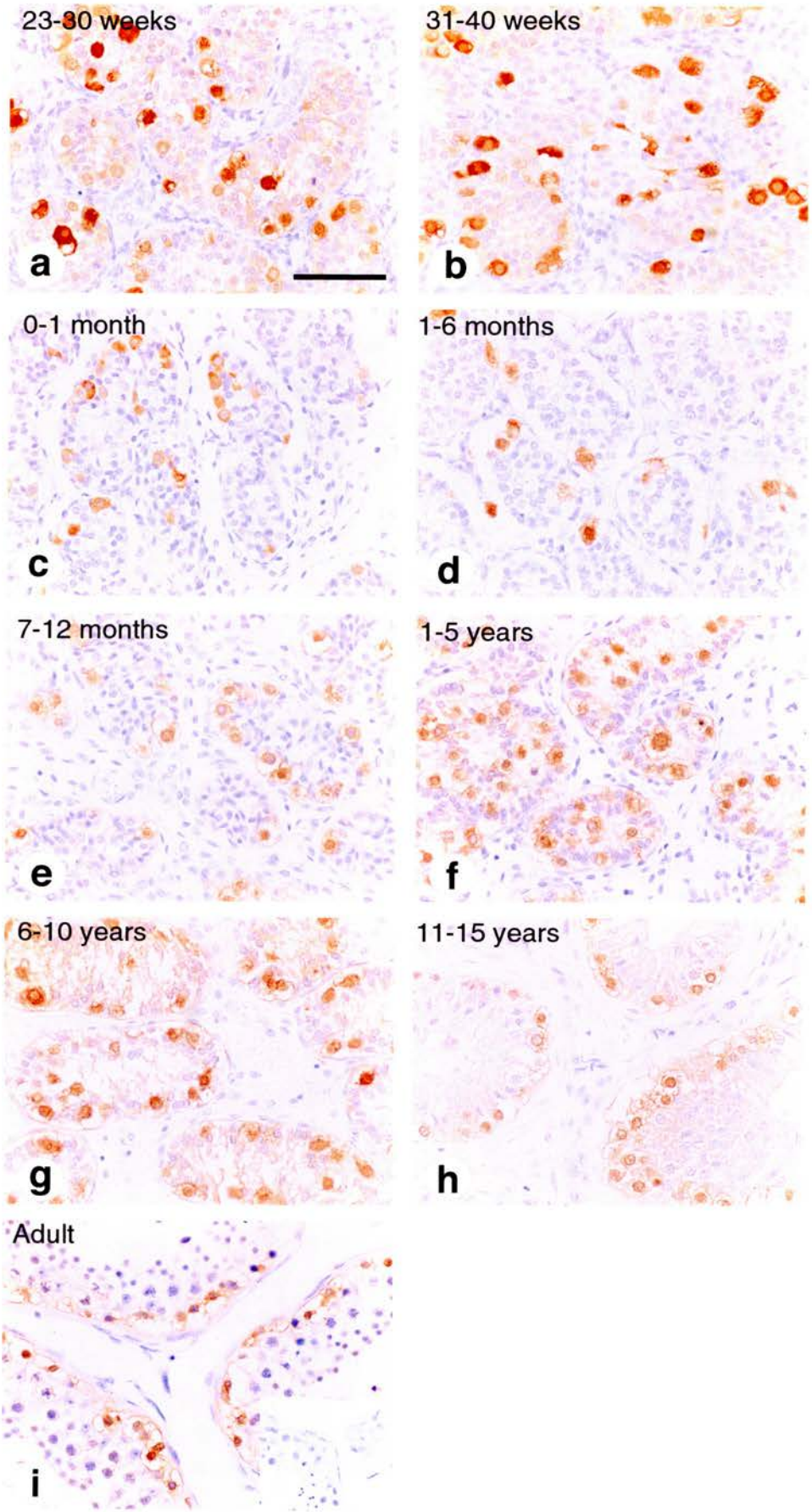
g
11-15 years
h
Adult
i

Figure 7.9. MAGE-57B immunoexpression: MAGE-57B immunoexpression was evident at all ages of development. Immunoexpression of MAGE-57B was most intense during foetal life, panels (a) and (b), and subsequently decreased in intensity during infancy and early childhood, panels (c) to (e). MAGE-57B immunoexpression was intense during mid childhood, panels (f) and (g) and less intense in the peripubertal specimen, panel (h). Immunoexpression in the peripubertal specimen also appears to be localized to the spermatogonia and early spermatocytes (h). A healthy adult control is shown for comparison in which MAGE-57B immunoexpression was confined to spermatogonia and early spermatocytes, with negative inset (i). Scale bar denotes 50 μm.


23-30 weeks
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g
11-15 years
h
Adult
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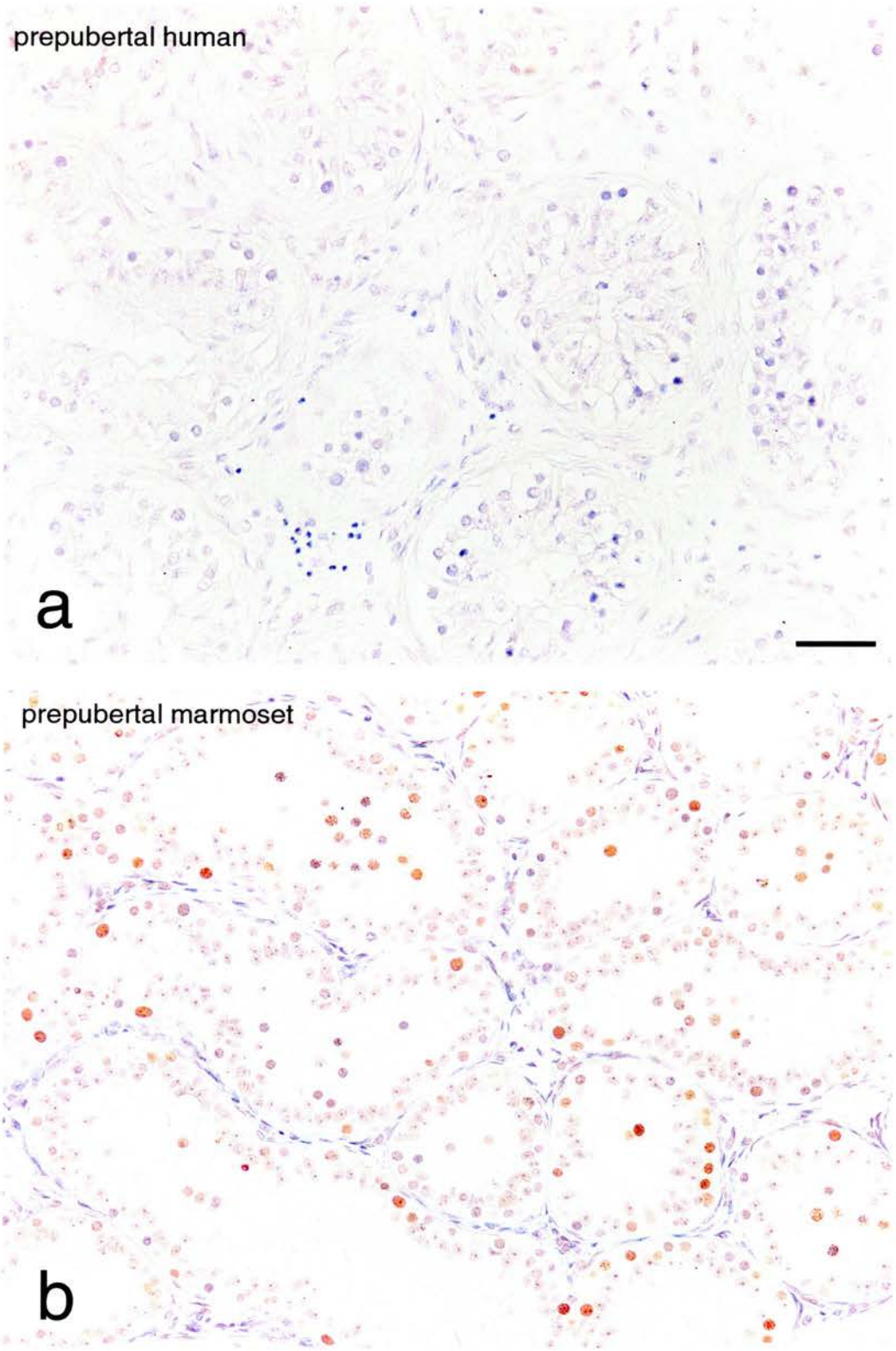
Figure 7.10. PCNA immunoexpression: PCNA immunoexpression was almost negligible in prepubertal human tissues, panel (a), in contrast to the abundant immunoexpression evident in prepubertal marmoset tissues, panel (b). Scale bar denotes 50 μm.


prepubertal human
a
prepubertal marmoset
b

7.4. Discussion

This study, to the best of my knowledge, is the first detailed immunohistochemical study to have investigated the development of the testis in the prepubertal human. There is growing evidence to suggest that the prepubertal human testis may be 'quietly active' as opposed to truly quiescent[33-35,161,183-186]. During childhood, there is Leydig cell development with an increase in absolute number[187] and functional activity, as evidenced by an increase in intratesticular[188] and spermatic vein levels of testosterone[189] between 2-4 and 6-8yrs of age, coinciding with night-time activation of pulsatile LH secretion[183-185]. Muller and Skakkebaek have shown that testicular volume increases significantly during childhood, consistent with a 3-fold increase in germ cell number[35]. Studies have also shown that the germ cells themselves undergo a degree of maturation to form spermatocytes, and occasionally spermatids, during childhood but these germ cells subsequently degenerate due to a lack of maturation or functional support from the Sertoli and Leydig cells[188]. These changes are reported to occur at least 4-5 years before the onset of puberty.

Immunohistochemical studies in marmosets have demonstrated that there is significant testicular activity detectable during mid childhood[33]. In the marmoset, the onset of puberty at around 50-60 weeks of age is associated with a rise in blood testosterone levels. Evaluation of functional development of Sertoli cells and Leydig cells, based on immunoexpression of SGP-2 and AR, and 3β-HSD respectively, demonstrated activity at an age of 35 weeks, when the testis is considered to be

quiescent, and in advance of the pubertal rise in blood testosterone levels, at 50-60 weeks[33]. This compelling evidence reinforces the tenet that the testis is not quiescent during childhood.

The marmoset was chosen as a primate model for the human, because it exhibits a comparable pattern of testicular development: it descends its testes into the scrotum by birth, exhibits neonatal proliferation of Sertoli cells and a testosterone surge followed by infantile 'quiescence'[33,181]. In addition, the adult marmoset testis has been shown to exhibit an organization of spermatogenesis that approaches that in the human in contrast to other commonly used primate models such as the rhesus monkey[177]. If comparable changes could be demonstrated in the prepubertal human testis it would validate the use of the marmoset as a model for the human.

Demonstration of cell division and maturation in the relatively 'quiescent' human testis during childhood, similar to the marmoset, may help us understand the susceptibility of the prepubertal human testes to damage from cytotoxic chemotherapy and radiotherapy. Kelnar *et al* have clearly demonstrated that the marmoset testis undergoes active development during childhood, considerably in advance of the clinical onset of puberty[33]. In an attempt to elucidate the mechanism of this childhood development, and possibly enhance our understanding of the susceptibility of the prepubertal testis to cytotoxic therapy, they studied the effect of treating these animals for a period during early childhood (25-35weeks) with a GnRH antagonist. The early activation of testicular function observed during mid

childhood, at 35 weeks, was largely abolished with GnRH antagonist treatment, indicating that these changes are essentially gonadotrophin dependent. Interestingly, there was no significant difference in PCNA-labelling index of spermatogonia between the GnRH antagonist treated and untreated groups, implying that GnRH antagonist therapy had no impact on germ cell proliferation but did impact on their survival. This latter observation has important implications when considering GnRH antagonist intervention therapy as a strategy for preserving testicular function in children undergoing treatment for childhood cancer. Clearly, furthering our understanding of the gonadotrophin independent mechanisms of germ cell proliferation and differentiation is essential. From this it is hoped to be able to explain why the prepubertal testis is susceptible to damage from cytotoxic cancer therapy. Moreover, using this primate model to develop methods of protecting spermatogenesis or preserving germ cells during cancer therapy would have enormous therapeutic implications for the young boy facing sterilising treatment for childhood cancer.

The findings in the marmoset studies would imply that functional development of testicular cells is likely to be initiated at some point during childhood. Based upon immunoexpression of a number of protein markers of testicular cell development in the marmoset, these have been applied to human tissue specimens to enable us to establish whether comparable changes in expression of these markers occur during human testicular development. Evaluation of Sertoli cell development and maturation was made using immunoexpression of the protein markers AR, SGP-2,

inhibin α and βB. Based upon the absence of immunoexpression of AR, SGP-2, inhibin βB, essentially throughout childhood, with the exception of weak immunoexpression of AR in the specimen from a 12yr old patient, this may suggest that the human testis is relatively quiescent during childhood, in contrast to the findings of earlier activity reported in the marmoset[33]. Similarly, lack of functional activity was observed in Leydig cells during childhood, based upon immunoexpression of 17α-OH and 3β-HSD. This is again in conflict with the earlier functional activity before the onset of puberty suggested by studies in the marmoset and also in boys[33,187-189]. Identification of proliferating germ cells with procedures used in the marmoset proved to be equally challenging. PCNA has been used as a means of identifying 'non-quiescent' cells in the marmoset during childhood, i.e. cells in the cell-cycle. In the marmoset, at 35 weeks of age, only germ cells, not Sertoli cells, are proliferating and therefore immunoexpress PCNA, enabling quantification of germ cell development. PCNA was not expressed in any of our specimens from children, in contrast to evidence of abundant proliferation demonstrated in the marmoset studies[33]. In this study, germ cells were positively identified using immunoexpression of the MAGE-57B antigen, throughout all ages of childhood, although no assessment of the differential expression within the different stages of germ cell development was made. Quantitative evaluation of the increasing number of germ cells relative to testicular volume is not possible to determine in our studies given the lack of available information regarding testicular volumes. However, studies are currently underway to express the number of germ

cells relative to the number of Sertoli cells present at any give age. In order to attempt to make some attempt at quantification of testis cell development studies are underway using point-counting techniques to express the percentage of germ cells relative to Sertoli cells. This will be achieved using a double immunohistochemical staining technique with MAGE-57B and GATA-4 to differentially identify germ cells and Sertoli cells respectively.

Our preliminary findings of testicular development in the prepubertal human are not in keeping with the level of activity suggested by studies in the marmoset. A number of reasons may be proffered to explain these discrepancies. Firstly, it may be premature to draw conclusions from this study, particularly for the older subjects of peripubertal age, based upon the paucity of specimens. Secondly, although immunohistochemistry conditions were optimised for human tissues, and demonstrated immunoexpression of the various protein markers in the specimens from the very young patients, the absence of immunoexpression from the older subjects may reflect inadequate techniques. Perhaps more importantly, the human testicular specimens were obtained from a tissue archive, which was not specifically designed to ensure maximum preservation of the tissue specimens, i.e. optimum antigen preservation, to a standard equal to that of the marmoset or adult human control specimens. With increasing testicular volumes the significance of the inadequate fixation procedures may have had a greater adverse impact on preservation of the antigens under investigation. Fixation issues are particularly of concern when 'absence of immunoexpression' occurs, as is the case in the present

series comparing prepubertal human with prepubertal marmoset testes. Absence of immunoexpression could result from a 'true absence' or from lack of antigen preservation, with these two opposing explanations being very difficult to distinguish. There are, however, some indicators from the present studies, for example, it was clear from inspection of H & E stained slides of the testes sections from boys aged 8-12 years that more germ cells were present, including some spermatocytes in the specimens from the oldest boys. Yet none of these samples exhibited PCNA-staining of the germ cells, even though it is established that most of these cells should be immunopositive, based on studies of the adult human testis and of the prepubertal marmoset testis[33]. This suggests quite strongly that there has been failure of PCNA preservation. However, this conclusion cannot be generalised, as it was equally clear that some antigens were still detectable, including the presence MAGE-57B in germ cells. 17α-OH and 3β–HSD were used as markers of steroidogenic activity in Leydig cells. Steroidogenic activity was clearly demonstrated in the very preterm infants with both 17α-OH and 3β–HSD staining immunopositive. During infancy some evidence of functional activity was demonstrated by positive immunoexpression of 3β–HSD only. The disappearance of 17α-OH may reflect loss of antigen expression or an earlier switching off of this antigen. Both markers were absent throughout childhood, including the older specimens of peripubertal age, when we would have expected to see some activity as demonstrated by inhibin α, again this may reflect lack of antigen preservation. Even then, if the marmoset model was a true reflection of the human we would have

expected evidence of functional activity at an earlier age, from mid childhood onwards. Before definitive conclusions can be drawn from this study about the applicability of marmoset studies as a model for the human a number of steps require to be taken. Clearly, recruitment of larger number of specimens from the older age bracket of children is essential and such studies are currently in progress. Furthermore, we need to explore alternative markers for identifying cells, particularly functional Leydig cell activity and germ cell proliferation. Unfortunately, as these specimens are obtained from a tissue archive it will not be possible to circumvent the problems of inconsistencies and possibly inadequacies, in the fixation procedures.

It is appreciated that our current studies evaluating the development of the testis in prepubertal humans is in the preliminary stages and at present it is not yet possible to definitively conclude whether the marmoset provides an appropriate model for development of the human testis during childhood. As this work is still in progress, we are hopeful that we will be able to answer these questions in the near future.

Chapter 8

Ethical and legal issues

8. Ethical, legal and practical considerations

Harvesting gonadal tissue and its future use is an exciting new area of gamete biology which raises a wide range of unresolved ethical and legal issues that must be addressed before embarking on any clinical programme[28,8,190-192]. These include issues relating to the safety of the tissue harvesting, subsequent use and possible implications for the progeny, together with the legal constraints enforced by the HFEA relating to gamete storage and manipulation, and the common laws defining validity of consent in the event of any such procedures becoming available. Each of these issues is addressed in this chapter and recommendations for future practice are discussed.

It is undisputable that harvesting and subsequent use of gonadal tissue is still in it's infancy but as it offers the potential for paternity to vulnerable young individuals, every effort will be made to pursue such developments. However at present, it remains undeniably experimental and as such, creates an area of uncertainty and unprecedented challenges, which must be addressed. The controversial issues arising from these experimental procedures, or more accurately clinical research, are twofold; firstly, they involve the use of reproductive, or potential reproductive, material, and secondly, they relate to children. A number of legal and ethical constraints govern both any work relating to mature reproductive tissue and research in children. Unfortunately, the pace of technological advances has outstripped the laws implemented to protect society from unwanted manipulation of the human

germline. The potential benefit of these developments to children with cancer could not have been predicted and consequently such a legacy may inadvertently hamper progress in this area if current legislation is not revised.

8.1. The legal framework

8.1.1. Consent for storage of human reproductive tissue

The field of assisted reproduction is governed by the statute in the UK and is under the jurisdiction of the Human Fertilisation and Embryology Authority (HFEA Act, 1990), which dictates strict guidelines on the requirements for informed consent with respect to the storage of gametes and embryos and their subsequent use[193]. The HFEA grants licences to individuals for certain procedures involving gametes. Proxy consent is specifically excluded and there is a requirement to provide written and verbal information and an offer of independent counselling[194]. The exclusion of substituted consent may be subject to further consideration under the UK Human Rights Act 1998, if this proves to be a hindrance in genuine cases of freezing of genetic material[194,195]. It is the responsibility of the licence holders to ensure that informed consent is obtained before embarking upon the cryopreservation procedure. The provider(s) of the gametes or embryos always retains the right to the control of their genetic material, the fate of which can be altered by a change in the consent at any time they choose to do so.

A gamete is defined by the HFEA to be 'a reproductive cell, such as an ovum or spermatozoon, which has a haploid set of chromosomes and which is capable of

taking part in fertilisation with another of the opposite sex to form a zygote'[197]. According to the Tanner classification of pubertal development, boys are capable of producing 'gametes' from Tanner stage 2 onwards. In practice this would mean that storage of testicular material from a boy who has reached at least Tanner stage 2 would require consent for storage from the individual, and the centre would require a licence to store the material. For postpubertal boys the preferred specimen is one produced by ejaculation using masturbation. However, sperm may be retrieved by alternative methods as discussed in chapter 7 (Table 7.3). For peripubertal boys, at least Tanner stage 2, who are deemed to have testicular tissue sufficiently mature to be considered as capable of producing gametes, the harvesting of tissue remains entirely experimental but the issues relating to consent for storage of tissue are as for the older boys. Difficulties of consent arise when boys have reached a stage at which sperm storage is physically possible but they are not deemed legally competent. In addition consent for storage of the material cannot be given by proxy, as stated by the HFEA and this is discussed further in the next section. In contrast, testicular tissue could be harvested from prepubertal boys provided that the parents give valid consent and the procedure is in the child's best interests. Until recently, the storage of immature testicular tissue from prepubertal subjects did not require a licence. However, recognition of advances in the field of fertility preservation and assisted reproduction has led to the necessary introduction of changes in the health service. In particular, the handling and storage of immature gametes now falls under the Department of Health Code of Practice for Tissue Banks, which came into force in April 2003[191,196].

8.1.2. Consent for harvesting gonadal tissue

Clinical research is regulated by a code of practice, which dictates that the field of research must be scientifically sound and in the patient's best interest, determined to be so by stringent ethical review. To enable the research to proceed any further the patient is then obliged to give informed consent. It is the issue of consent that is fundamental to the debate. Any research intervention, which may in some circumstances be potentially harmful must be ethically and legally acceptable and valid consent obtained. To be valid, consent must be informed, voluntarily obtained, and given by a competent person. In practice, it may be difficult to satisfy these criteria, especially in patients with cancer. The information necessary for parents and children to make an informed choice about fertility preservation is inevitably complex and its comprehension cannot be guaranteed. Legal competence to consent requires that the individual giving it is able to understand the information given, believes that it applies to them, retains it, and uses it to make an informed choice. In the case of children, consent is proxy, given by the parent or guardian. Legal competence may be diminished by parental anxieties about their child's illness. Such issues may also involve consideration of a future, which neither they, nor the child, can envisage or have discussed. In addition, therapeutic imperatives may limit the time available for discussion, which in turn imposes constraints on the voluntariness of the consent. Issues surrounding the safety of the procedures, both with regard to the harvesting of the tissue and it's storage, raise further concern and, together with the uncertainty relating to any potential success of future use of the material, makes obtaining informed consent unrealistic.

8.1.2.1. Consent – a two-stage process

Some of these practical difficulties may be alleviated if obtaining consent is considered as a continuum, which can be divided into two stages, with part one involving harvesting and cryopreservation of the tissue, and part two involving subsequent use of the tissue. Future use of the tissue also raises a number of issues for both autotransplantation or in vitro maturation of the tissue. Autotransplantation carries a potential risk of reintroducing cancer cells into the patient, while in vitro maturation would involve the development of mature gametes and subsequent use with ART, and consequently regulation by the HFEA. Clearly, subsequent use of the tissue would require separate consent from the patient, who would have reached a stage of sufficient maturity to be able to make decisions relating to his/her fertility. Issues relating to the use of the tissue in the event of the death of the patient must also be discussed and, in accordance with the regulations of the HFEA, it would be likely to take the path of destroying the tissue in the event of the patient's death.

The removal of testicular tissue is an invasive procedure that carries an element of risk, a risk that is augmented in individuals whose health is already compromised by their disease. The subsequent reimplantation of stored tissue may carry as yet unquantified risks with the potential of reactivating disease in individuals in remission. Given our current lack of knowledge surrounding the issues of safety and future use, it is questionable whether even competent individuals can give fully informed consent. Reiteration of the experimental nature of these procedures is necessary during any discussions with the patient and the family.

8.1.2.2. Who gives the consent?

The need and the difficulties arising from obtaining informed consent from an adult have been established but what are the issues for children? Obtaining consent in young people remains a contentious issue, which is confusing for the patient, the parent and the medical professionals involved in promoting the child's health and enabling them to exercise, where possible, their autonomy regarding reproductive issues. Normally, consent models pertain to treatment but cryopreservation of sperm or testicular tissue from a mature male is preventative and normally requires no surgical intervention. In the UK, adolescents over the age of 16 years of age in Scotland, and 18 years of age in England, may give consent to treatment in accordance with the Family Law Reform Act 1969 s8, while for younger children, minors, consent is generally obtained by proxy[198,199]. In exception to this, legally valid consent from minors can be obtained if their doctor considers that they are competent to make an informed and wise decision[200,201]. More recently, in the USA the concept of assent has been developed. Early adolescents are considered 'to assent' (or, its converse, dissent) when they have sufficient competence to have some appreciation of a procedure, but not enough to give fully informed consent. The age of assent is currently estimated as being 12[201-203].

Specifically for adolescents a 'family rule' model of consent has been developed[204]. This is a framework for obtaining ethical consent for medical interventions in children. The rule proposes that informed consent in children can be regarded as shared between children and their families, the balance being determined by implicit,

developmentally based negotiations between the child and the parent. Involvement of the family may help provide support for the adolescent making the decision. It will provide an opportunity for the family to discuss such delicate matters more openly and may even serve to bring the family closer together and unite them together to face something positive during such a devastating time. Providing informed consent or assent, is a tremendous responsibility for the adolescent who will be facing a number of complex issues at this difficult time, and involving the parents may allow open discussion and explanation of complex language to play a guiding role in helping the adolescent to give informed consent but retaining a degree of autonomy.

In practice there are three scenarios

i. In sexually mature minors, sperm may be produced and consent obtained in accordance with the Gillick principle and cryopreserved in a licensed centre.
ii. If the patient, at least Tanner stage 2, is unable to produce a sample by masturbation, sperm may be surgically retrieved and stored, provided that the patient is able to give written informed consent.
iii. The patient is pre-pubertal and therefore the testicular tissue does not contain 'gametes' as defined by HFEA, the legal and practical considerations falls under the Department of Health Code of Practice for Human Tissue Banking (enforced since April 2003)[196]. Under these circumstances, parental consent is required for the surgical procedure.

As early as 1970 the World Health Organisation recognised that it was a fundamental right of every individual to have freedom from organic disorders, disease and deficiencies that interfere with sexual and reproductive function. Therefore not to offer storage, by denying choice at a later stage in life, may be an infringement of this right. Success in animals, however, does not guarantee applicability in humans and optimisation of the methodology and refining of storage techniques should be restricted to specialist centres with experience in the cryobiology of reproductive tissue.

8.2. Recommendations for best code of practice

Harvesting and storage of ovarian cortical tissue from girls and young women before gonadotoxic chemotherapy has been available in a number of centres since the mid-1990s and, more recently, a few centres report the storage of testicular tissue[205]. The Royal College of Obstetricians and Gynaecologists has provided a report from a working party on the storage of ovarian and prepubertal testicular tissue. This provides standards for best practice in the cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures and requirements for safe storage[79].

In December 2002 an international conference was held in Cambridge, the specific aim of which was to develop an ethically acceptable strategy for the practice and research related to preserving fertility in children and adolescents being treated for cancer[28]. From this meeting a consensus statement was drawn up which made a

number of recommendations. Integral to these recommendations were the design and implementation of well constructed research strategies, confined to a finite number of specialist centres, with centralization of data and rapid dissemination of the results. In turn, these protocols and results should be subject to rigorous review to ensure high standards for collection procedures and storage of material. At both a research and clinical level this would involve multidisciplinary teamwork with multicentre collaboration, dictating optimum communication to ensure the best interests of the child are met. It was also recommended that prospective studies be set up to gather data about the impact of current treatment strategies on fertility outcomes in prepubertal children treated for cancer to facilitate further our understanding of the gonadotoxic impact of chemotherapy and radiotherapy in the hope that future treatment regimens may be modified accordingly. The experimental nature of this work makes it essential to ensure that clinical and research practice develops in a phased and co-ordinated manner, as outlined in Table 8.1.

Table 8.1 Development strategy for research into germ cell harvest and storage.

Phase 1
Develop a consensus regarding treatment-related risks of germ cell damage
Develop a consensus regarding risks to the child associated with germ cell harvest
Phase 2
Develop methods of prospective data collection aimed at registering germ cell tissues, collection, and conditions of storage
Develop a register of patients at risk of infertility
Phase 3
Monitor success rates of the use of stored germ cell material and the fertility rates of those registered
Phase 4
Careful follow-up of the offspring of children born following assisted reproduction

Chapter 9

Conclusions

9. Conclusions

As treatment for childhood cancer has become increasingly successful, adverse effects on reproductive function are assuming greater importance. Treatment of childhood cancer with chemotherapy and radiotherapy may damage testicular function resulting in impaired spermatogenesis and temporary or permanent infertility in adulthood. In this study testicular function and semen quality were investigated in 33 survivors of childhood cancer. It has been shown that the treatment of cancer during childhood was associated with a significant risk of subsequently impaired spermatogenesis, with 30.3% of this population being azoospermic and 18.2% being oligozoospermic. Moreover, in those men who do have surviving spermatogenesis after treatment, it is commonly compromised, with reductions being observed in ejaculate volume, sperm concentration, sperm motility and the proportion of morphologically normal sperm. Only 33.3% of this group of 33 male childhood cancer survivors had completely normal semen quality by conventional criteria. However, the sperm produced do not appear to carry a greater burden of damaged DNA compared with the healthy population, suggesting that assisted conception treatment is a safe option for these men.

Traditionally, determining the impact of chemotherapy and radiotherapy on gonadal function has involved clinical assessment of pubertal development and semen analysis in males. Earlier detection of gonadal damage has been hampered by the lack of a sensitive marker of gonadal function in prepubertal children. The role of

inhibin B as a marker of early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer was investigated. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed deleterious effect. Inhibin B changed earlier and appeared to be a more sensitive marker of gonadal damage than FSH or LH. It was concluded that inhibin B may be a useful addition to sensitive measurements of FSH in the evaluation of early gonadotoxic effects of chemotherapy in prepubertal children. Further studies are planned, combining inhibin B with FSH, LH and sex hormone measurements, to assess long-term reversibility and delayed effects, particularly as the children approach and progress through puberty.

For prepubertal boys, fertility preservation through semen cryopreservation is not an option and consequently, attention is focusing on the development of techniques that might preserve or restore fertility potential in boys being subjected to gonadotoxic cancer therapy. In rats, it has been shown that some germ cells survive cytotoxic therapy and that the resulting azoospermia is a consequence of the inability of those spermatogonia that are present to proliferate and differentiate. Suppression of the hypothalamic-pituitary-gonadal (H-P-G) axis facilitates recovery of spermatogenesis following such cytotoxic treatment. Investigation of whether suppression of the H-P-G axis in men rendered azoospermic by treatment for childhood cancer might restore spermatogenesis was undertaken, using both semen analysis and testicular biopsy as end points. In men treated with sterilising radiotherapy and chemotherapy for childhood cancer, effective gonadotrophin suppression with medroxyprogesterone

acetate for at least 3 months did not result in restoration of spermatogenesis. The absence of histological evidence of spermatogonial stem cells in testicular biopsies from these men before and after suppression suggests complete ablation of the seminiferous epithelium and irreversible infertility.

Understanding the vulnerability of the prepubertal human testis to cytotoxic damage is compounded by the dearth of data describing normal testicular development in the prepubertal human. Based on immunohistochemical studies in marmosets, a primate that exhibits a similar developmental profile to the human male, it has been shown that significant testicular development occurs during childhood long before the clinical onset of puberty. If we can establish that cell activity does occur in the 'quiescent' testis in boys and is comparable to changes shown in the marmoset, it will validate use of the marmoset as a model for the human in this instance and give encouragement to the possibility of using this primate model to develop a method of protecting spermatogenesis in boys undergoing cancer therapy prior to puberty. Preliminary studies to investigate the development of the prepubertal human testis confirmed testicular cell activity in the foetal and neonatal periods and infancy comparable to that shown in the marmoset. However, to date development during mid childhood and early puberty has proved to be somewhat discordant with the marmoset studies. It is too premature to definitively conclude that marmoset and human testicular development are dissimilar, as a number of explanations have been proffered to explain the discrepancies, including suboptimal tissue fixation and/or antigen preservation in the human tissues.

9.1. Future objectives

It is incumbent upon oncologists to facilitate appropriate counselling of patients at risk of subfertility as part of their routine care. Preservation of fertility before treatment must be considered in all young patients at high risk of subfertility. Limitation of radiation exposure, by shielding of the testes, should be practiced where possible and sperm banking should be offered to all sexually mature boys at risk of infertility. Semen can be stored before sterilising chemotherapy and radiotherapy for use at a later date, thus preserving fertility.

For prepubertal boys, lacking in haploid gametes, there are no options currently available to preserve fertility and harvesting of testicular germ cells before sterilising cancer therapy is the most promising option. However, any potential strategies must be considered entirely experimental. Capitalising on the unique properties of the spermatogonial stem cell, testicular tissue could be harvested before treatment and cryopreserved for use at a later date. The functional capacity of the testes appears to be preserved and autotransplantation of the stem cells in rats has been shown to re-initiate spermatogenesis and to permanently restore fertility. Where fears of reintroducing tumour cells may limit applicability of this approach in humans, *in vitro* maturation and use with assisted reproduction techniques is a realistic alternative.

It is in the area of stem cell biology that our future research will focus. Integral to this study will be the development of stem cell culture to enable further study of stem

cell biology and ultimately develop techniques for *in vitro* proliferation and/or maturation. Our objective is to optimise stem cell isolation and purification techniques and to develop safe and efficient germ cell transplantation methods in mice. If autologous germ cell transplantation is to be considered in oncology patients, ensuring transplantation of tumour free cells is paramount. Preliminary steps would involve the development of methods to isolate and cryopreserve spermatogonial stem cells from mice testes and to establish stem cell purification procedures. Identification of stem cell markers will be undertaken and stem cell culture techniques will be developed in mice. Any new understanding from mice would subsequently be applied to marmosets, and with this in mind, procedures for germ cell transplantation in the marmoset are in progress and showing some success.

Harvesting gonadal tissue and its future use is an exciting new area of gamete biology which raises a wide range of unresolved ethical and legal issues that must be addressed before embarking on any clinical programme. I have explored the inherent ethical, legal and scientific dilemmas that must be addressed before embarking on any clinical programme of gonadal tissue harvesting, and I have presented the current recommendations for considering harvesting of gonadal tissue in boys undergoing treatment for childhood cancer.

The importance of the late effects of treatment are receiving increasing recognition and in the UK strategies for long-term follow up are currently being explored[3]. Consideration of fertility preservation is a quality of life issue at a time of intense

stress for young patients and their families. Nevertheless, in our experience, open discussion is embraced and often potentially therapeutic for the vulnerable family facing treatment for cancer. Discussion of fertility issues at the time of diagnosis provides the family with the reassurance that they can look forward to long-term survival for their child, and a future when these issues will become important. It is also important to be aware of other quality of life issues in this field that are likely to emerge as the numbers of treated cancer survivors enter old age. In particular, the age-related decline in testosterone levels in most men (and it's attendant sequelae) may be advanced or exacerbated in male cancer survivors, based on the evidence that some men show evidence of compensated Leydig cell failure[17-19,53]. Long-term follow-up is essential for these men to enable early recognition of problems and, where possible, effective therapy instituted to optimise quality of life.

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Appendix

Semen quality and spermatozoal DNA integrity in survivors of childhood cancer: a case-control study

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Summary

Background Treatment of childhood cancer can result in impaired spermatogenesis. Intracytoplasmic sperm injection (ICSI), however, can enable men to achieve fatherhood, and has focused attention on gamete integrity in men with oligozoospermia. Our aim was to assess testicular function in survivors of childhood cancer.

Methods We assessed testicular function in 33 survivors of childhood cancer and 66 age-matched controls. The median age at diagnosis and at the start of the trial was 10·0 years (range 2·2–16·9) and 21·9 years (16·5–35·2), respectively. We assessed pubertal staging, measured plasma sex steroid hormone concentrations, and analysed semen quality, including spermatozoal DNA integrity.

Findings Ten (30%) individuals were azoospermic and six (18%) oligozoospermic (sperm concentration $<20\times10^6$/mL). Sperm concentration was significantly lower in the non-azoospermic group than in controls (median $37{\cdot}1\times10^6$/mL, IQR $19{\cdot}7\times10^6$ to $89{\cdot}9\times10^6$, *vs* $90{\cdot}7\times10^6$/mL, $50{\cdot}5\times10^6$ to $121{\cdot}5\times10^6$; p=0·002). In the non-azoospermic cancer survivor group, inhibin B concentrations were lower than in controls (mean 153·3 ng/L, SEM 17·8, *vs* 223·7 ng/L, 8·8; p<0·001), and FSH concentrations were higher (6·6 U/L, 0·9, *vs* 3·2 U/L, 0·2; p<0·001). Only 11 (33%) survivors of childhood cancer had normal semen quality. There was no significant difference in sperm DNA integrity between the non-azoospermic and control groups (9%, 5–13, *vs* 11%, 7–16; p=0·06).

Interpretation Sperm concentration is reduced after treatment for cancer. However, the sperm produced seems to carry as much healthy DNA as those produced by the healthy population, suggesting that assisted conception can be considered as a treatment option for these men.

Lancet 2001; **360:** 361–367. Published online July 9, 2002
http://image.thelancet.com/extras/01art7042web.pdf

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Introduction

70% of individuals survive childhood cancer, and this proportion continues to increase. As such, attention is being focused on the lasting morbidity associated with radiation and chemotherapy treatment.[1] A frequent and psychologically traumatic late complication of cancer treatment is infertility. Cytotoxic chemotherapy drugs, especially alkylating agents, can produce long-lasting or permanent damage to the germinal epithelium, resulting in oligozoospermia or azoospermia.[2–8] The germinal epithelium is also sensitive to radiotherapy, and doses as low as 1·2 Gy can result in permanent sterility.[9] Recovery from surviving germ cells can happen but is unpredictable and often takes a long time.[7,8] Leydig cells, with their slower rate of turnover, are more resistant to gonadotoxic therapy, resulting in preservation of androgen production even when patients are infertile.[5]

Advances in techniques of assisted reproduction, especially intracytoplasmic sperm injection (ICSI), have enabled some men with oligozoospermia to become fathers.[10,11] Concerns have been raised, however, about the safety of ICSI,[12] since whether or not spermatozoa from men with impaired spermatogenesis carry abnormal genetic information is unkown.[13–15] Data on the health of offspring born after ICSI are broadly reassuring,[16] though there are no data on the health of children born to fathers whose deficit in semen quality is a specific consequence of potentially mutagenic treatment.[17] Results of studies in animals have shown that exposure of the male germ line to chemotherapy agents can disrupt spermatozoal DNA and result in deleterious effects on embryo development.[18] Awareness of the importance of the integrity of sperm DNA for accurate transmission of genetic material to the offspring has necessitated the development of new techniques to assess sperm characteristics in more detail.[19]

Our aim was to investigate testicular function and semen quality in survivors of childhood cancer.

Methods

Patients

We searched the oncology database at the Royal Hospital for Sick Children, Edinburgh, for all male survivors of childhood cancer aged older than 16 years, and identified 51 individuals between December, 1999, and June, 2001. We invited 45 of these men to participate in the study, and excluded six because they no longer lived in the area (five) or were on antidepressant medication (one). Six of the 45 men declined and six did not reply to the invitation. The 18 men who did not participate in our study were comparable for age, diagnoses, age at diagnosis, treatment regimens, and disease-free survival. 33 men participated in the study. For each study participant, we recruited two age-matched controls (n=66). The volunteers were recruited by means of advertisement in local media and through hospital out-patient clinics, and selected on the basis of the absence of any clinical evidence, on history or physical examination, of reproductive health problems. The Lothian Paediatric and Reproductive Medicine research ethics subcommittee approved the study, and all patients provided written informed consent.

Study protocol

We assessed pubertal maturation according to the Tanner criteria, and measured testicular volume with the Prader orchidometer.[20] We noted the mean volume of the two testes as the individual's testicular volume. We identified concentrations of luteinising hormone (LH), follicle stimulating hormone (FSH), and testosterone in venous blood samples (20 mL) with an automated immunoassay analyser (Bayer Immuno 1, Bayer, Newbury, Berkshire, UK), and measured inhibin B concentrations in serum from venous blood samples that had been centrifuged at 2000 *g* for 10 min and stored at –20°C.[21] Semen samples were collected in a room adjacent to the laboratory, by masturbation into sterile, wide-mouthed non-toxic containers, after an abstinence period of at least 48 h. The samples were analysed for ejaculate volume, sperm concentration, motility, and normal morphology, according to WHO protocols.[22] Throughout the study, the laboratory was subject to external quality control.

We measured the extent of DNA fragmentation in spermatozoa with the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) technique, according to the general methodology of Sun and colleagues,[19] modified as follows. We obtained an unselected population of cells by mixing an aliquot of each sperm sample with Biggers-Whitten-Whittingham medium[23] containing 20 nmol N-(2-hydroxyethyl) piperazine-N′-(2-ethan sulfonic acid) (Invitrogen, Paisley, UK) and 0·3% human serum albumin solution, and centrifuged at 500 *g* for 5 min. After decanting the supernatant, we resuspended the pellet of spermatozoa in 2 mL of phosphate buffered saline (Sigma-Aldrich, Gillingham, UK) and centrifuged at 500 *g* for 5 min. This step was repeated and the spermatozoa were subsequently fixed in 1% formaldehyde (Sigma-Aldrich) in phosphate buffered saline for 60 min at room temperature. The fixed sperm concentration was adjusted to 20×10^6 cells/mL with a Neubauer haemocytometer. We centrifuged the sample at 500 *g* for 5 min and washed it in phosphate buffer saline. The fixed sperm were resuspended in 100 µL prewash buffer, containing single strength One-Phor-All buffer (Amersham Pharmacia Biotech, Buckinghamshire, UK) and 0·1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature. We centrifuged out the sperm from the buffer at 500 *g* for 30 min and resuspended in 50 µL of TdT buffer, containing 3 µmol biotin-16-dUTP (Roche Diagnostics, Lewes, UK), 6 µmol dATP (Amersham Pharmacia Biotech), and 1 IU/µL of TdT enzyme (Amersham Pharmacia Biotech), and incubated at 37°C for 1 h. After washing in phosphate buffer saline, we resuspended the fixed permeabilised sperm in 100 µL of staining buffer, consisting of 0·1% Triton X-100 (in distilled water) and 1% streptavidin/fluorescin conjugate (Calbiochem-Novabiochem, Nottingham, UK), and incubated the sample in the dark at 4°C for 30 min. The stained cells were spun at 500 *g* for 5 min and resuspended in 500 µL phosphate buffer saline to give a concentration of about 1×10^6 cells/mL. For negative controls, we omitted the enzyme terminal transferase from the reaction mixture. For positive controls, we treated the samples with 0·8 IU/µL DNase I (Roche Diagnostics) for 15 min at room temperature before incubation with the TdT buffer.

Patient	Diagnosis	Chlorambucil total dose (g/m²)	Procarbazine total dose (g/m²)	Vinblastine total dose (g/m²)	Ifosfamide total dose (g/m²)	Cyclophosphamide total dose (g/m²)	Cytarabine total dose (g/m²)	Radiotherapy: area applied/ total dose (Gy)	Sperm concentration (×10⁶/mL)
1	ALL	..	..	..	..	..	2·76 (2·00)	Cranium/total body irradiation 24/14·4	0
2	ALL and testis relapse	..	..	..	..	..	2·93 (2·00)	Cranium/testis 18/24	0
3	HD	0·90 (0·67)	16·80 (11·20)	134·00 (96·00)	..	..	..	None	0
4	HD	0·50 (0·50)	8·40 (8·40)	79·20 (72·00)	..	..	..	None	0
5	HD	0·50 (0·50)	8·40 (8·40)	72·00 (72·00)	..	..	..	Mediastinum 30	0
6	HD	0·50 (0·50)	10·00 (8·40)	86·40 (72·00)	..	..	..	Upper mantle 30	0
7	HD	0·34 (0·50)	6·30 (8·40)	54·00 (72·00)	..	..	..	Medias/neck 35	0
8	B cell NHL	..	..	..	..	..	2·20 (2·00)	Cranium/total body irradiation 6/14·4	0
9	Ewing's sarcoma	..	..	..	139·20 (87·00)	..	..	Fibula/lung 45/12·5	0
10	Ewing's sarcoma	..	..	..	165·60 (84·00)	..	..	None	0
11	Teratocarcinoma (maxilla)	..	..	..	..	..	..	Maxilla 60	0·55
12	HD	0·80 (0·67)	14·00 (11·20)	112·00 (96·00)	..	..	..	None	4·55
13	Relapsed ALL	..	..	..	..	5·67 (4·20)	2·70 (2·00)	Cranium 24	5·75
14	Ewing's sarcoma	..	..	..	132·60 (102·00)	4·30 (3·30)	..	Radius 55	12·45
15	ALL	..	..	..	..	..	1·20 (1·00)	None	14·75
16	ALL	..	..	..	..	..	4·80 (3·80)	Cranium 18	19·10
17	ALL	..	..	..	..	..	..	Cranium 15	20·29
18	Pineal germinoma	..	..	..	..	..	..	Cranium 35	20·30
19	ALL	..	..	..	..	..	–(>3) NI	Cranium 24	21·00
20	B cell NHL	..	..	..	..	..	2·28 (2·00)	None	24·65
21	Osteosarcoma	..	..	..	..	..	..	None	33·75
22	Wilms' tumour	..	..	..	..	..	..	None	37·10
23	ALL	..	..	..	..	..	NI	Cranium 24	42·00
24	Ewing's sarcoma	..	..	..	138·60 (126·00)	..	..	None	53·00
25	ALL	..	..	..	..	0·78 (1·20) NI	1·30 (2·00) NI	Cranium 24	66·25
26	ALL	..	..	..	..	0·45 (0·60) NI	NI	Cranium 24	77·00
27	ALL	..	..	..	..	..	..	Cranium 18	85·00
28	Medulloblastoma	..	..	..	..	..	..	Cranium/spine 55/35	94·83
29	ALL	..	..	..	..	..	..	Cranium 21	103·50
30	ALL	..	..	..	..	..	1·86 (2·00)	Cranium 18	113·25
31	Ewing's sarcoma	..	..	..	144·00 (106·00)	..	..	None	125·25
32	ALL	..	..	..	..	..	..	Cranium 18	145·00
33	ALL	..	..	..	..	..	..	Cranium 18	230·00

ALL=acute lymphoblastic leukaemia. HD=Hodgkin's disease. NHL=non-Hodgkin lymphoma.

Table 1: **Diagnosis and exposure to potentially gonadotoxic treatment by sperm count of all study patients**

	Long-term survivors of childhood cancer		
	Azoospermic (n=10)	Non-azoospermic (n=23)	Controls (n=66)
Characteristics			
Age (median, range) (years)	19·5 (16·5–25·3)	22·4 (17·6–35·2)	20·8 (18·0–36·3)
Smoking habit			
Yes	4 (40%)	5 (22%)	25 (38%)
No	6 (60%)	18 (78%)	41 (62%)
Alcohol consumption (units/week) (%)			
0	1 (10%)	6 (26%)	5 (8%)
≤10	5 (50%)	3 (13%)	6 (9%)
11–20	1 (10%)	11 (48%)	29 (44%)
≥21	3 (30%)	3 (13%)	26 (39%)
Pubertal staging			
Tanner	5	5	5
Testicular volume (median, IQR) (mL)	10 (7·4–12)*	17·5 (15–21·25)	20 (15–23)

Data are number (%) unless otherwise indicated. *p<0·001 for azoospermic group compared with other two groups.

Table 2: **Testicular function in long-term survivors of childhood cancer and in controls**

We analysed the samples with an Epics XL flow cytometer (Beckman Coulter Corporation, Buckinghamshire, UK) with a 15 mW argon ion laser operating at 488 nm. We measured green fluorescence with the FL1 detector at 525 nm. The flow rate during analysis was controlled at 200 events/s, and we analysed 10 000 events in each sample. Light-scatter and fluorescence data were obtained at a fixed gain setting in the logarithmic mode. Debris were gated out on the basis of forward scatter versus side scatter dot plot, by drawing a region enclosing the cell population of interest, and 10 000 events were collected. We processed the data with an IBM compatible computer installed with System II, version 1.0 (Beckman Coulter Corporation). We identified the proportion of labelled sperm in each sample.

Seven of the ten men identified on semen analysis as being azoospermic had a testicular biopsy done under general anaesthetic, to exclude a diagnosis of obstructive azoospermia. We fixed the specimens in Bouins and stained them with haemotoxylin and eosin. The presence of spermatogonial stem cells was assessed.

Statistical analysis

We did statistical analysis with SPSS (version 10.0). Analysis of variance was done on the endocrine data (non-parametric) and ejaculate volume (Kruskal-Wallis test), the data common to all three groups. We did pair-wise tests to study differences between the groups, if such differences existed. We did *t* tests and Mann-Whitney *U* tests to compare the differences between patient groups and controls with respect to endocrine data and semen data, respectively. For comparisons that involved all three patient groups, three pair-wise two-sample tests were done and Bonferroni corrections applied to the p values. A p value less than 0·05 was judged significant.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

The 33 men had a median age of 21·9 years (range 16·5–35·2). Their median age at diagnosis of cancer was 10·0 years (2·2–16·9), and they had a median disease-free survival time of 11·6 years (0·3–24·4). The underlying malignancies included acute lymphoblastic leukaemia (15), Hodgkin's disease (six), Ewing's sarcoma (five), non-Hodgkin lymphoma (two), brain tumours (two), Wilms' tumour (one), osteosarcoma (one), and teratocarcinoma (one). Table 1 shows the patients' diagnoses and details of the potentially gonadotoxic chemotherapy and radiotherapy received. Table 2 shows characteristics of patients and controls.

Of the 33 patients, ten were azoospermic. Five of these individuals had received treatment for Hodgkin's disease with the alkylating agents chlorambucil, procarbazine, and vinblastine (table 1), all of which are gonadotoxic. Two of the azoospermic patients had been treated with ifosfamide for Ewing's sarcoma, two had received total body irradiation, and one had received direct testicular irradiation (table 1). Of the ten azoospermic patients, seven were prepubertal at diagnosis, providing cogent evidence that the prepubertal testis is not afforded protection from cytotoxic insult.

Six (18%) patients were oligozoospermic (sperm concentration $<20\times10^6$/mL), with severe oligozoospermia ($<2\times10^6$/mL) in one individual. In controls, however, oligozoospermia was seen in only three (5%) individuals. Only one of the six men treated for Hodgkin's disease with an alkylating agent-based regimen showed preservation of spermatogenesis (sperm concentration $4{\cdot}55\times10^6$/mL). Three of the oligozoospermic patients had been treated with Medical Research Council Protocols, UKALL II, III, and X, which consisted of combination chemotherapy, including, vincristine, prednisolone, 6-mercaptopurine methotrexate, cytarabine, and cyclophosphamide. Oligozoospermia was seen in one of the five patients treated for Ewing's sarcoma, for whom treatment included ifosfamide and cyclophosphamide. The remaining oligozoospermic patient (sperm concentration $0{\cdot}55\times10^6$/mL) did not receive treatment with agents expected to be gonadotoxic, and the reason for impaired spermatogenesis remains unknown.

Sperm concentration in the non-azoospermic group of individuals treated for cancer was significantly lower than the sperm concentration of controls (table 3, figure 1). Nine (29%) of the patients were asthenozoospermic

	Long-term survivors of childhood cancer			95% CI azoospermic *vs* controls	95% CI non-azoospermic *vs* azoospermic	95% CI non-azoospermic *vs* controls
	Azoospermic (n=10)	Non-azoospermic (n=23)	Controls (n=66)*			
Semen characteristics						
Abstinence (h)	62 (40·5–137·8)	60·4 (36–82·5)	73·5 (59·3–91·5)	..	..	..
Ejaculate volume (mL)	1·9 (1·5–2·3)	2·5 (2·1–3·5)	3·4 (2·5–5·1)	−2·7 to −0·7†	−1·5 to 0·2	−1·7 to −0·3†
Sperm concentration ($\times10^6$/mL)	0	37·1 (19·7–89·9)	90·7 (50·5–121·5)	..	..	−67 to −15†
Progressive motility (%)	..	56·3 (44·4–64·7)	61·9 (55·5–69·1)	..	..	−15·1 to −0·6‡
Normal morphology (%)	..	6·5 (3·7–7·6)	9·3 (6·3–11·0)	..	..	−4·6 to −1·3†
TUNEL damage (%)	..	8·8 (5·1–12·6)	11·4 (7·2–16·3)	..	..	−5·3 to 0·2

Data are mean (IQR). *n=64 for TUNEL assay. †= p<0·01. ‡=p<0·05.

Table 3: **Semen quality in long-term survivors of childhood cancer and in controls**

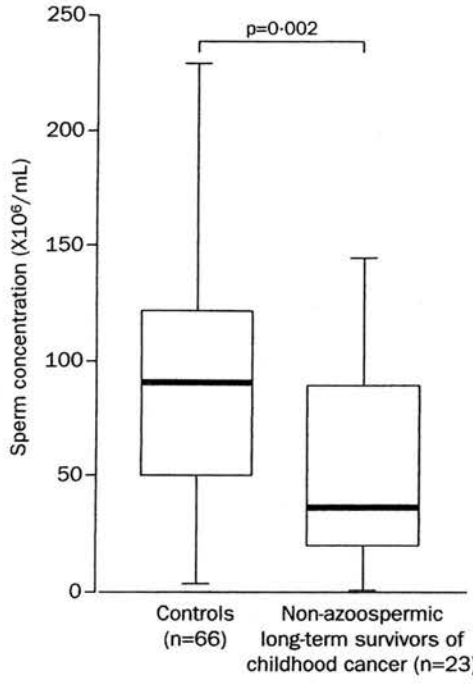

Figure 1: **Sperm concentration in non-azoospermic long-term survivors of childhood cancer and in controls**
Median, IQR, and maximum and minimum values are shown.

(progressive motility <50%) compared with ten (15%) controls. Median progressive motility for the non-azoospermic group was significantly less than for the control group (table 3). The proportion of sperm with normal morphology was also significantly less in the non-azoospermic group than in controls (table 3). From our population of 33 male survivors of childhood cancer only 11 (33%) men had a normal semen analysis as defined by

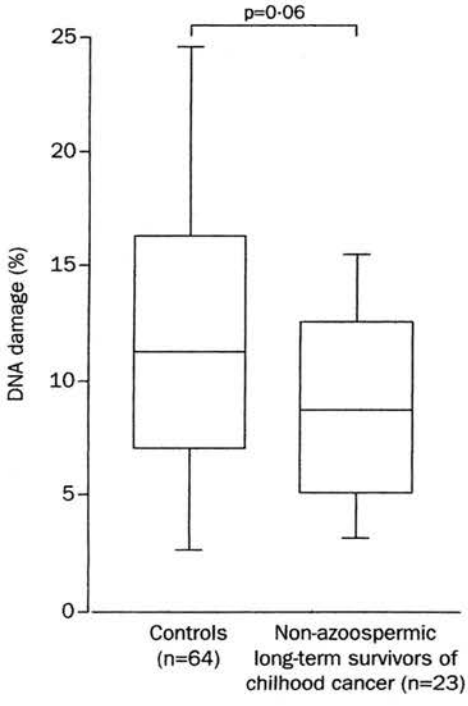

Figure 2: **Sperm DNA integrity in long-term survivors of childhood cancer and in controls**
Median, IQR, and maximum and minimum values are shown.

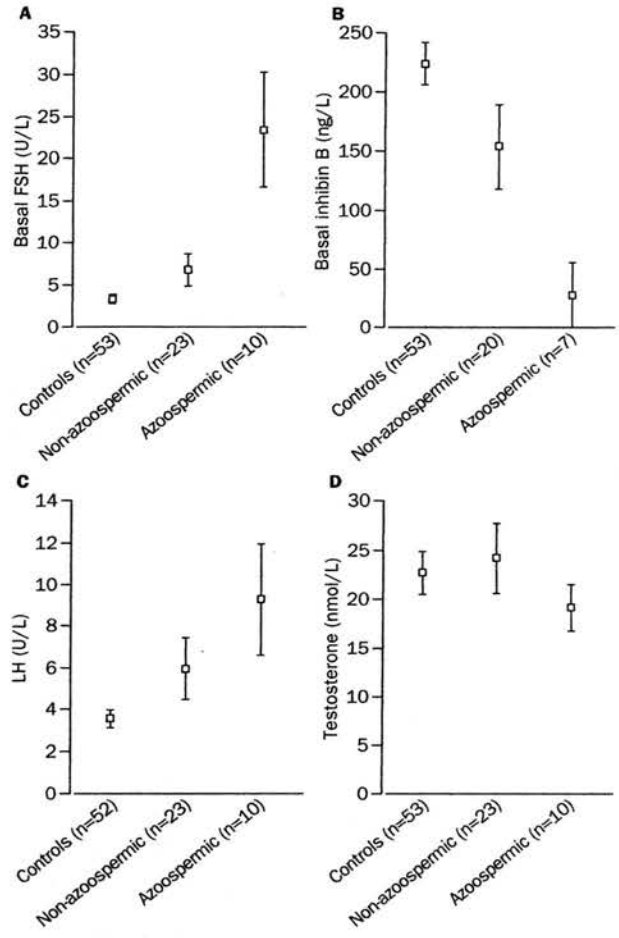

Figure 3: **Comparison of values for follicle stimulating hormone (FSH) (A), inhibin B (B), luteinising hormone (LH) (C), and testosterone (D) in the control group, and in non-azoospermic and azoospermic cancer survivors**
Mean and SEM are shown. Reference ranges for FSH and LH are 1·5–9·0 U/L and for testosterone 10–30 nmol/L. There is no established reference range for inhibin B.

WHO compared with 55 (83%) in the control group. There was no correlation between either age at diagnosis or time out from treatment and sperm concentration in this group of cancer survivors who were non-azoospermic ($p=0·109$ and $p=0·516$, respectively). There was a difference in ejaculate volume between the three groups ($p<0·001$). Ejaculate volume was significantly reduced in the azoospermic and non-azoospermic groups compared with controls ($p=0·002$ and $p=0·006$, respectively).

We assessed the integrity of the spermatozoal DNA with the TUNEL assay and the results are shown in table 3. There was no significant difference between DNA fragmentation in the non-azoospermic group and controls (table 3, figure 2).

Testicular biopsies from seven of the ten men with azoospermia on semen analysis showed a Sertoli-cell only picture in all instances, thus excluding obstructive azoospermia.

Analysis of variance for the endocrine measurements, FSH, LH, testosterone, and inhibin B, showed a significant difference for the FSH, LH, and inhibin B values for the three groups ($p<0·001$), but no significant difference was seen between the groups for testosterone concentrations. Pair-wise tests were subsequently done to assess the differences between the groups, with Bonferroni corrections applied.

Basal serum concentrations of FSH were significantly higher in the azoospermic group than in the non-azoospermic (mean difference −16·6, 95% CI −22·0 to −11·3, p<0·001) and control (−20, −23·1 to −17·1, p<0·001; figure 3A) groups. The concentrations of FSH were also significantly greater in the non-azoospermic group than in the controls (−3·4, −4·8 to −2·1, p<0·001; figure 3A). Inhibin B concentration was barely detectable in the azoospermic group, and was significantly lower in that group than in both the non-azoospermic (129·3, 61·2 to 190·5, p<0·001) and control groups (198·9, 146·6 to 245·9, p<0·001; figure 3B). Inhibin B concentrations were also significantly lower in the non-azoospermic group than in the control group (69·6, 34·6 to 106·3, p<0·001; figure 3B). Serum concentrations of LH were significantly greater in the non-azoospermic and azoospermic groups than in the controls (−2·4, −3·5 to −1·2, and −5·6, −7·1 to −4·2, respectively, p<0·001 in both instances, figure 3C). Testosterone concentrations were within normal limits in all three groups, with no significant differences between the groups (figure 3D).

Discussion

Our results indicate that treatment of childhood cancer is associated with a high risk of impaired spermatogenesis in adulthood. Moreover, in men in whom spermatogenesis continues after treatment, production is generally compromised, with reductions arising in ejaculate volume, sperm concentration, sperm motility, and in the proportion of morphologically normal sperm. Reassuringly, however, the integrity of the genomic DNA carried by the gametes of men treated for cancer seems unaffected. Our findings suggest that the reduction in sperm number is directly attributable to the cytotoxic effects of cancer therapy.

Fertility and sexual function are the principal life-style concerns in more than 80% of men successfully treated for cancer,[24] yet a substantial proportion of survivors invited refused to take part in our study. Cancer survivors are less likely to have children for several reasons other than treatment-induced infertility, including an inability to form relationships, fear of relapse of their disease, and the prospect of leaving a child parentless.[24]

Cytotoxic treatment for childhood cancer should minimise unwanted side-effects without compromising survival. Where there is equal efficacy between regimens, the effect of drugs on reproductive function should be considered when devising the most appropriate therapy.[25] Our data confirm the sterilising effects of treatment for Hodgkin's disease with a standard regimen, ChlVPP (chlorambucil, vinblastine, procarbazine and prednisolone).[7,8] The ABVD (adriamycin, bleomycin, vinblastine and dacarbazine) combination, which does not contain alkylating agents or procarbazine, is much less gonadotoxic, resulting in temporary azoospermia in 33% of patients and oligozoospermia in 21%, with full recovery after 18 months reported in all patients.[26] Consequently, with the introduction of hybrid regimens, three cycles of ABVD with three cycles of ChlVPP or MOPP (mechlorethamine, vincristine, procarbazine, and prednisolone), gonadotoxicity could be greatly reduced.

Three of the sterile men in our study had been treated with either fractionated total body irradiation or testicular irradiation. Although recovery has been reported after several years, the high doses administered in these treatments make recovery unlikely.[9] Furthermore, as a prerequisite of recovery, some stem cells must survive, and in these three men, as indeed in all seven men biopsied, stem cells were absent.

Ifosfamide, an analogue of cyclophosphamide, potentially gonadotoxic. Five of our patients had receive treatment that included ifosfamide for Ewing's sarcom Of these, two had a normal semen analysis, one wa oligozoospermic, and the remaining two patients wer azoospermic. The three patients with sperm present i their ejaculate had been treated more than 11 yea previously, compared with the azoospermic patients wh had completed treatment 4 and 7 months previously. Th two sterile patients were sexually mature at the time diagnosis and had provided semen samples fo cryopreservation before commencing cancer therapy, wit sperm concentrations of 124×10^6/mL and 128×10^6/mL This finding suggests that recovery of testicular functio with increasing time since treatment is a possibilit Continued, perhaps yearly, reassessment of seme analysis is warranted in azoospermic patients.

Normal semen quality, as defined by WHO, stipulate that sperm concentration must be greater tha 20×10^6/mL and progressive motility greater than 50%. According to these criteria, only a third of the survivors childhood cancer had a normal semen analysis, compare with most of the control group. Our results are similar t those reported by Lopez Andreu and co-workers.[27]

Oligozoospermia was observed in six (18%) of the long term survivor patients, compared with three (5%) in th control group. One of the patients, with sever oligozoospermia, had been treated with alkylating agent for Hodgkin's disease. Three of the oligozoospermi patients received treatment with standard protocols fo acute lymphoblastic leukaemia, which included cytarabin and in one patient cyclophosphamide. The doses cytarabine and cyclophosphamide received by these thre patients were significantly less than previously reported t be gonadotoxic doses[6] and could indicate individu susceptibility. Lendon and colleagues[28] studied th testicular histology of 44 boys treated for acut lymphoblastic leukaemia and noted a severely depresse tubular fertility index, <40%, in 18 individuals, indicativ of germinal epithelial damage. In a follow-up study b Wallace and colleagues,[4] semen analysis in seven of thes patients with severe depression of tubular fertility inde who had been off treatment for a median of 10·8 yea (range 5·5–15·9), reported azoospermia in four the patients and full recovery of spermatogenesis i three (sperm concentration $>20\times10^6$/mL)—ie, previou chemotherapy treatment with cyclophosphamide an cytarabine impaired gonadal function, which improve with increasing time after treatment in some patients.

Inhibin B mediates non-steroidal negative feedbac from the testes, reflecting the number of spermatozo produced and regulating FSH secretion.[29,30] Inhibin secretion in the adult requires the presence of germ cells. Inhibin B concentrations were barely detectable in th azoospermic patients, in whom the germ cells wer destroyed, despite preservation of Sertoli cells, a confirmed on testicular biopsy. This finding provide further evidence for the essential role of the germ cel Sertoli cell interaction for the production of inhibin B and confirms the value of inhibin B as a non-invasiv marker of spermatogenesis after cytotoxic therapy.

The sperm concentration in the non-azoospermic grou was significantly less than that of the general population Although azoospermia after gonadotoxic chemotherap has been widely investigated there are few data abou sperm concentrations in those patients in whom spermatogenesis is preserved. Rautonen and co-workers report median sperm concentrations of 67×10^6 mL (rang $0{\cdot}01\times10^6$ to 425×10^6) in 27 patients treated for various

childhood haematological and solid cancers. Sperm concentrations of $20{\cdot}4\times10^6$ mL ($3{\cdot}2\times10^6$ to 43×10^6) were reported in a small study cohort (four) treated for acute lymphoblastic leukaemia (one) and non-Hodgkin lymphoma (three), and 31×10^6 mL ($4{\cdot}5\times10^6$ to 100×10^6) in eight long-term survivors of acute lymphoblastic leukaemia, indicating a general trend towards a lower sperm concentration.[32,33] In view of the fact that intact spermatogenesis requires the presence of stem cells, which are capable of self-renewal in addition to differentiation, the discrepancy in sperm concentrations between the non-azoospermic and control groups is difficult to reconcile. The non-azoospermic group clearly retains a population of intact stem cells capable of undergoing normal spermatogenesis, yet the surviving stem cells do not seem to repopulate the pool to produce sperm concentrations comparable with the general population. This deficit could reflect a more complex picture, emphasising the important role of Sertoli cells in supporting a finite number of stem cells. If there has been subtle damage to the germinal epithelium, involving loss of Sertoli cells, remaining Sertoli cells might already be functioning to full capacity. This possibility is suggested by the subnormal inhibin B concentrations and corresponding rise of serum FSH concentrations in the non-azoospermic group compared with the control population. This finding is analogous to data showing a decline in plasma inhibin B concentrations, directly in proportion to Sertoli cell numbers, after unilateral orchidectomy in rhesus monkeys.[34] In a physiological setting, where the negative feedback control system that regulates the testes is operational, Sertoli cell number is the primary determinant of circulating inhibin B concentrations.[34]

Ejaculate volume was reduced in the non-azoospermic and azoospermic groups compared with the control group, a finding which is unlikely to be attributable to retrograde ejaculation, obstruction, impaired autonomic innervation, or incomplete specimen collection. Testosterone concentrations were normal in both groups of patients, making testosterone deficiency an unlikely cause of decreased ejaculate volume. Damage to the prostate is unlikely, since only three of the patients had radiotherapy treatment involving the pelvis. Whether chemotherapy plays a part is uncertain. In a study[31] of 55 long-term survivors treated with chemotherapy or radiotherapy for various childhood cancers, 18 (33%) were reported to have a low ejaculate volume.

The mutagenic potential of cancer therapy might confer a risk to the fetus conceived with gametes produced after cancer therapy, although current epidemiological data suggest that offspring of cancer survivors do not have an increased incidence of congenital abnormalities or cancer relative to the general population.[35] However, an important concern is that these results are largely based on offspring arising from natural conception, and the consequences of circumventing the natural selection processes of fertilisation involved by means of ICSI, are unknown.[15] There is at least the hypothetical possibility of injection of abnormal spermatozoa carrying abnormal genomic DNA with the potential to increase congenital and other abnormalities among offspring.[19] Techniques to assess spermatozoal DNA integrity have been developed,[14,19] and it has become clear that men from subfertility clinic populations, with abnormalities of the conventional criteria of semen quality, also have increased amounts of damage to the genomic DNA in their gametes. Even among normal populations, sperm chromatin damage has been linked with impaired fecundity.[36] Spermatozoal DNA damage does not preclude pronucleus formation at ICSI,[13] and abnormal DNA within the male gamete is detectable in the early embryo.[37] Concern thus arises in the case of childhood cancer survivors, given that the capacity of ionising radiation and some chemicals to induce transmissible genetic damage in the germ cells of laboratory mammals has been clearly shown.[38] Thus far, evidence on the safety of ICSI has been largely based on its use in populations of men with deficits in spermatogenesis unrelated to potentially mutagenic cancer treatment. This evidence has been broadly reassuring with respect to health risks to the offspring,[16,39] although it is limited by restricted length of follow-up available. We have shown that although the conventional criteria of semen quality are frequently abnormal in long-term survivors of childhood cancer, the sperm produced do not seem to carry a greater burden of damaged DNA. This observation goes some way to providing reassurance about the use of ICSI, which will circumvent the problems associated with severe oligozoospermia and asthenozoospermia, and offer cancer survivors the possibility of paternity in adulthood.

Contributors

A B Thomson participated in study design, patient recruitment, semen and TUNEL analysis, analysis of results, and writing and editing of the report. A J Campbell participated in recruitment of controls, semen and TUNEL analysis in controls, and editing of the report. D S Irvine, C J H Kelnar, and R A Anderson participated in the study design, analysis of results, and editing of the report. W H B Wallace participated in the study design, patient recruitment, analysis of results, and writing and editing of the report. W H B Wallace, C J H Kelnar, and D S Irvine obtained funding.

Conflict of interest statement

None declared.

Acknowledgments

We thank M P Shaw and A E Thomas, at the Royal Hospital for Sick Children, Edinburgh, for assistance in recruitment of patients for the study and for allowing patients to be studied, respectively; and R Elton for his assistance with statistical analysis. The study was funded by Pharmacia Corporation and the Child Growth Foundation.

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Human Reproduction Vol.17, No.7 pp. 1715–1723, 2002

Investigation of suppression of the hypothalamic–pituitary–gonadal axis to restore spermatogenesis in azoospermic men treated for childhood cancer

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BACKGROUND: Does suppression of the hypothalamic–pituitary–gonadal (HPG) axis restore spermatogenesis in men rendered azoospermic following treatment of childhood cancer? METHODS: Seven men with azoospermia secondary to treatment for childhood cancer, median age (range), 22.2 (18–25.3) years, aged 10.4 (4.4–13.3) years at original diagnosis, participated. Each subject underwent semen analysis and testicular biopsy, followed by treatment with medroxyprogesterone acetate (MPA), 300 mg i.m. repeated after 12 weeks, with 800 mg testosterone pellets s.c. on day 1 to suppress the HPG axis. Hormone and semen analysis was performed every 6 weeks for 48 weeks. A second testicular biopsy was performed at week 48. RESULTS: Before HPG axis suppression, mean ± SEM plasma LH was 9.0 ± 1.8 U/l, testosterone 17.9 ± 1.5 nmol/l and FSH 22.4 ± 4.4 U/l. Median (range) venous plasma and seminal plasma inhibin B levels were 10.0 (7.8–35) and 11.2 (7.8–770) ng/l respectively. During HPG suppression, FSH and LH levels were undetectable for ≥12 weeks followed by a gradual return to pretreatment concentrations by 48 weeks. All men remained azoospermic at study completion and complete absence of germ cells on biopsies was demonstrated by immunocytochemistry for all specimens pre- and post-HPG axis suppression. CONCLUSIONS: HPG axis suppression with MPA–testosterone for ≥12 weeks did not restore spermatogenesis in azoospermic men treated with gonadotoxic radiotherapy and chemotherapy for childhood cancer.

Key words: cancer/childhood/hormone suppression/spermatogenesis/testis

Introduction

Advances in the treatment of childhood cancer mean that most children can realistically hope for long-term survival. With a 70% overall survival rate, the prevalence of long-term survivors in the young adult population is now estimated to be 1 in 1000 (Wallace, 1997). Of increasing concern among survivors is the deleterious impact that chemotherapy and radiotherapy has on future fertility irrespective of pubertal status at the time of treatment (Heikens *et al.*, 1996; Mackie *et al.*, 1996; Howell and Shalet, 1998; Waring and Wallace, 2001; Thomson *et al.*, 2002). For prepubertal boys, fertility preservation through semen cryopreservation is not an option and consequently, attention is focusing on the development of techniques that might preserve or restore fertility potential in boys being subjected to gonadotoxic cancer therapy.

A number of approaches to this problem have been investigated, based on the idea that suppression of spermatogenesis might protect the normally rapidly dividing germ cell population from damage. Suppression of the rat hypothalamic–pituitary–gonadal (HPG) axis by administration of the GnRH analogue, goserelin, before and during chemotherapy with procarbazine, enhanced recovery of spermatogenesis (Ward *et al.*, 1990). Similarly, protection of spermatogenesis in rats subjected to treatment with procarbazine, cyclophosphamide and radiotherapy has been demonstrated using a number of hormones including testosterone alone (Delic *et al.*, 1986) or in combination with estrogen (Kurdoglu *et al.*, 1994), GnRH analogues in combination with testosterone (Pogach *et al.*, 1988) or the anti-androgen flutamide (Kangasniemi *et al.*, 1995; Meistrich *et al.*, 1995).

Furthermore, recovery from spermatogenic damage in rats induced by radiotherapy or procarbazine treatment has been shown to be enhanced by treatment with GnRH analogues or testosterone even when administered after the gonadotoxic agent (Pogach *et al.*, 1988; Meistrich and Kangasniemi 1997; Meistrich *et al.*, 1999). The mechanisms by which such hormonal manipulation offers protection or enhancement of recovery of spermatogenesis are unclear. Hormonal analysis following irradiation in rats has shown a marked increase in intratesticular testosterone levels and it has been postulated

Table I. Patient diagnoses and their exposure to gonadotoxic treatment

Patient	Diagnosis	Chlorambucil TD, g (g/m^2)	Procarbazine TD, g (g/m^2)	Vinblastine TD, g (g/m^2)	Cytarabine TD, g (g/m^2)	Radiotherapy TD (Gy)	Sperm concentration ($\times 10^6$/ml)
1	ALL	–	–	–	2.76 (2)	Cr/TBI 24/14.4	0
2	ALL/testis relapse	–	–	–	2.93 (2)	Cr/testis 18/24	0
3	HD	0.9 (0.67)	16.8 (11.2)	134 (96)	–	None	0
4	HD	0.5 (0.504)	8.4 (8.4)	79.2 (72)	–	None	0
5	HD	0.5 (0.504)	10 (8.4)	86.4 (72)	–	Upper mantle 30	0
6	HD	0.34 (0.504)	6.3 (8.4)	54 (72)	–	Medias/neck 35	0
7	B-cell NHL	–	–	–	2.2 (2)	Cr/TBI 6/14.4	0

ALL = acute lymphoblastic leukaemia; HD = Hodgkin's disease; NHL = Non-Hodgkin's lymphoma; TD = total dose; Cr = cranium; TBI = total body irradiation.

that suppression of the HPG axis promotes multiplication and differentiation of spermatogonia by lowering testosterone concentrations within the testis (Meistrich and Kangasniemi, 1997).

While there is significant evidence for the success of protection/recovery strategies in rats, clinical studies in man have to date been inconclusive (Johnson *et al.*, 1985; Waxman *et al.*, 1987; Masala *et al.*, 1997) and there have been no trials investigating the effects of post-gonadotoxic hormonal suppression. The present study has investigated whether suppression of the HPG axis in men rendered azoospermic by treatment for childhood cancer might restore spermatogenesis, using both semen analysis and testicular biopsy as endpoints.

Materials and methods

The study was approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee and all patients gave written informed consent.

Patients

A review of the oncology database at the Royal Hospital for Sick Children, Edinburgh for men rendered azoospermic secondary to treatment for childhood cancer, identified seven men aged 22.2 (18–25.3) [median (range)] years. All men were invited to participate in the study regardless of the underlying malignancy or cytotoxic therapy and all seven accepted. The median age at original diagnosis was 10.4 (4.4–13.3) years with a disease-free survival of 8.4 (3.3–14.7) years. The underlying malignancies were acute lymphoblastic leukaemia (n = 2), Hodgkin's disease (n = 4) and non-Hodgkin's lymphoma (n = 1). A summary of the patients' diagnoses with details of the gonadotoxic chemotherapy and radiotherapy received is given in Table I.

Clinical assessment and routine haematological and biochemical assessment was performed on each patient to ensure that there was no evidence of disease relapse or second primary malignancy, or other reason likely to impair spermatogenesis. None of the patients had a family history of impaired spermatogenesis.

Assessment of testicular function

Pubertal maturation was assessed according to the Tanner criteria and testicular volume (ml) was measured using a Prader orchidometer (Tanner and Whitehouse, 1976). The mean value of the two testes was taken to represent the subject's testicular volume. Venous blood samples were collected (20 ml), and LH, FSH and testosterone levels determined using an automated immunoassay analyser (Bayer Immuno 1, Bayer plc., Newbury, Berks, UK). Inhibin B was measured as previously described (Groome *et al.*, 1996), with the limit of assay sensitivity being 7.8 pg/ml. Semen samples were collected in a room adjacent to the laboratory, by masturbation into sterile wide-mouthed non-toxic containers, following an abstinence period of ≥48 h. Samples were centrifuged at 3000 *g* for 30 min and the pellet examined to confirm azoospermia (World Health Organization, 1999). Seminal plasma was stored at –70°C until assayed for inhibin B (Anderson *et al.*, 1998). Testicular biopsy under general anaesthetic was undertaken on all patients at the start of the study to exclude obstructive azoospermia. The specimens were fixed in Bouin's fixative and after routine processing and paraffin embedding, sections were cut at 5 μm and examined. A second biopsy of the same testis was performed at the end of the study.

HPG axis suppression

All men underwent a period of suppression of the HPG axis, designed to induce hypogonadotrophic hypogonadism with reduced intratesticular testosterone levels for a period of ~24 weeks, followed by a recovery period of 24 weeks. Following testicular biopsy, subjects were administered depot medroxyprogesterone acetate (DMPA, 300 mg i.m.; Pharmacia and Upjohn, Milton Keynes, UK) and testosterone pellets (4×200 mg s.c.; NV Organon, Oss, The Netherlands). Administration of DMPA was repeated 12 weeks later.

Subjects were reviewed at 6 weekly intervals throughout the 48 weeks of the study for clinical assessment, blood sampling and semen analysis.

Immunohistochemistry of testicular tissue

The objective of the immunohistochemical analysis was to investigate whether or not any germ cells, in any developmental stage, were present in the testes of patients before or after HPG suppressive treatment. This was achieved using immunoexpression of the MAGE-57B antigen and androgen receptor (AR). The MAGE-57B antigen is expressed in early germ cells, strongly in the spermatogonial and weakly in early spermatocytes (Aubry *et al.*, 2001). AR is expressed in the nuclei of all Sertoli cells but not in the nuclei of any germ cells that might be present (Saunders *et al.*, 1996).

Unless otherwise stated, all incubations were undertaken at room temperature. Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed in water. A temperature-induced antigen retrieval step was required for AR only. The sections were pressure-cooked in 0.01 mol/l citrate buffer, pH 6.0 for 5 min at full pressure, allowed to stand for 20 min, cooled in running tap water and washed twice in (5 min each wash) in Tris-buffered saline [TBS: 0.05 mol Tris–HCl, pH 7.4, 0.85% (w/v) NaCl]. Endogenous peroxidase activity was then blocked by immersing sections in 3% (v/v) H_2O_2 in methanol

Table II. Patient characteristics before hypothalamic–pituitary–gonadal suppression

Patient	Tanner stage	Testicular volume (ml) (adult >12 ml)	FSH (U/l) (ref range: 1.5–9)	LH (U/l) (ref range: 1.5–9)	Testosterone (nmol/l) (ref range: 10–30)	Inhibin B (venous) (ng/l)	Inhibin B (semen) (ng/l)
1	5	12	18.2	6.6	21.9	35.0	770
2	5	5	45.1	19	17.7	15.5	27.7
3	5	10	23.9	9.3	21.5	<7.8	14.2
4	5	10	23	8	20.5	<7.8	8.2
5	5	5.5	25.2	8.8	10.2	<7.8	<7.8
6	5	8	9.8	6.3	16.6	12.2	N/O
7	5	12	11.6	4.9	17.2	N/O	<7.8
Mean (SEM)		11	22.4 (4.4)	9.0 (1.8)	17.9 (1.5)		

N/O = values not obtained.

(both from BDH Laboratory Supplies, Poole, UK) for 30 min, followed by two 5 min washes in TBS. Sections were incubated for 30 min with the appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) to block non-specific binding sites. Normal swine serum (NSS) and normal rabbit serum (NRS) (both from Diagnostics Scotland, Carluke, UK) were used for AR and MAGE-57B respectively. Primary antibodies were added to the sections at the appropriate dilution in either NSS–TBS–BSA (for AR 1:2000: AR N-20, Santa Cruz Biotechnology SC0816, Santa Cruz, CA, USA) or NRS–TBS–BSA (for MAGE-57B: 1:50, source of antibody) and incubated overnight at 4°C in a humidified chamber. The sections were washed twice in TBS and then incubated for 30 min with anti-rabbit or anti-mouse horse-radish peroxidase-labelled polymer (EnVision: Dako, Ely, UK) for AR and MAGE-57B respectively. Sections were washed twice (5 min each) in TBS and immunostaining was developed using liquid diaminobenzidine (Dako) until staining was optimal, when the reaction was stopped by immersing sections in distilled water. The sections were counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody.

Immunostained sections were examined and a mean of 132 (range 40–252) tubular cross-sections were counted for each specimen pre- and post-HPG suppression. The sections were photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, UK) fitted with a digital camera (Kodak DCS330: Eastman Kodak, Rochester, NY, USA). Captured images were stored on a computer (G4; Apple Computer Inc., Cupertino, CA, USA) and compiled using Photoshop 5.0 before printing using an Epson Stylus 870 colour printer (Seiko Epson Corp., Nagano, Japan).

Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Science (SPSS Inc., Chicago, III) version 10.0. For each individual patient, hormone concentrations at the beginning and end of the study were compared using paired *t*-tests and $P < 0.05$ was considered significant.

Results

Clinical features

All seven men were Tanner stage 5 with reduced testicular volumes of 11 (5–12) ml [mean (range), Table II]. There were no changes in clinical features during the study and no changes in testicular volume.

HPG axis suppression

MPA–testosterone treatment was well tolerated and there were no adverse effects. One man reported increased libido during the first 12 weeks of the study. Pretreatment serum FSH levels were elevated, 22.4 ± 4.4 U/l (mean ± SEM, reference range: 1.5–9), in keeping with damage to the seminiferous epithelium (Table II). Serum LH concentrations pretreatment were 9.0 ± 1.8 U/l (reference range: 1.5–9) and testosterone 17.9 ± 1.5 nmol/l (reference range: 10–30), indicating compensated Leydig cell dysfunction (Table II). Serum and seminal plasma inhibin B concentrations were barely detectable in all but one subject (subject no. 1: 35 and 770 ng/l respectively; Table II).

FSH was suppressed to undetectable concentrations during MPA–testosterone treatment for 12 weeks, and remained partially suppressed during the subsequent 12 weeks (Figure 1). Thereafter, there was a gradual rise by weeks 42–48 to 19.5 ± 3.6 U/l, which was not significantly different from pretreatment concentrations. LH showed a similar pattern to FSH, with suppression to undetectable concentrations for 12 weeks, followed by gradual recovery to 8.9 ± 1.6 U/l at 48 weeks (Figure 1). There was no statistically significant difference between LH concentrations pretreatment and at 48 weeks.

Conversely, testosterone concentrations rose initially following MPA–testosterone administration to a peak of 29.7 ± 1.9 nmol/l at 6 weeks, close to the upper limit of the normal range. This was followed by a gradual decline to a nadir of 10.2 ± 0.2 nmol/l at 30 weeks, with a subsequent slight rise to 13.8 ± 1.9 nmol/l at weeks 42–48. The latter was not significantly different from the pretreatment concentration.

Serum inhibin B concentrations increased from 35 to 60.9 ng/l at 12 weeks in the subject with the highest inhibin B pretreatment, and rose from <15 ng/l to low but detectable concentrations (range 16–35 ng/l) in the five other subjects (venous serum reference range: mean 257, 95% CI 231–284 ng/l). Serum inhibin B concentrations fell towards

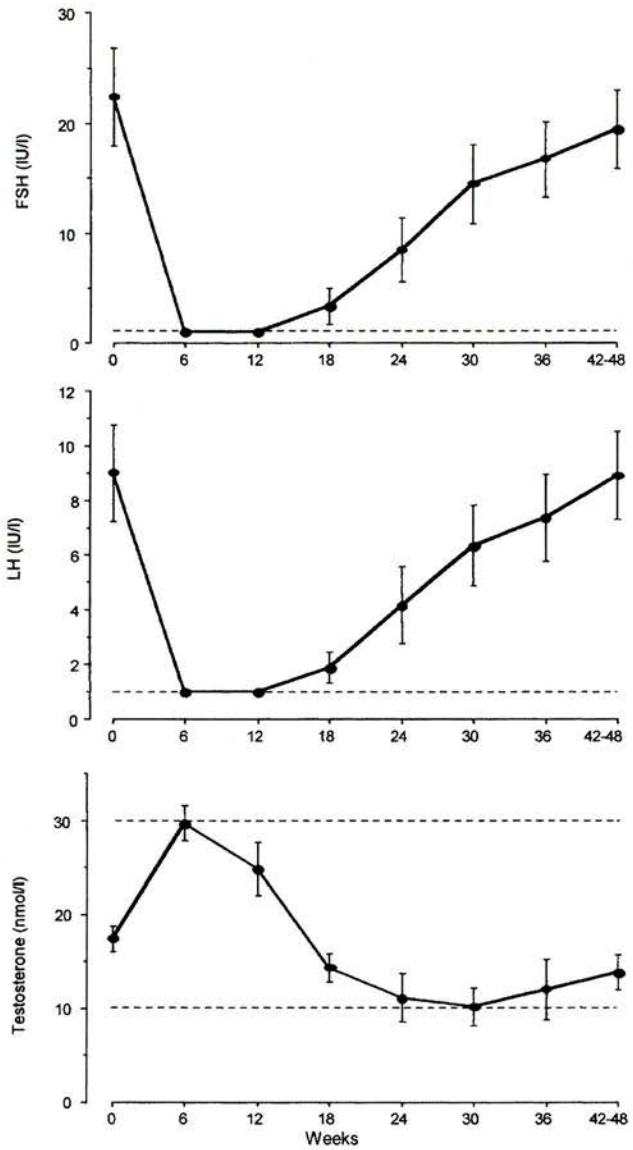

Figure 1. Serum concentrations of FSH, LH and testosterone in the seven patients before (time 0) and during combined treatment with medroxyprogesterone acetate and testosterone. Values are means ± SEM. Dashed lines show the limit of assay sensitivity (FSH, LH) or the upper and lower limits of the normal range (testosterone). Hormone levels at 42–48 weeks were not significantly different from pretreatment (week 0) values in each instance.

the end of the study, becoming undetectable in all subjects other than the individual with the highest pretreatment concentration at weeks 42–48. This same individual was the only subject with readily detectable seminal plasma inhibin B concentration pretreatment (770 versus <20 ng/l in the others, seminal plasma reference range: mean 2279, 95% CI 698–3864 ng/l) (Anderson and Sharpe, 2000). Seminal plasma inhibin B was not determined during MPA–testosterone treatment as the volume of the ejaculate was insufficient. In all subjects, seminal plasma inhibin B concentrations were undetectable at the end of the study.

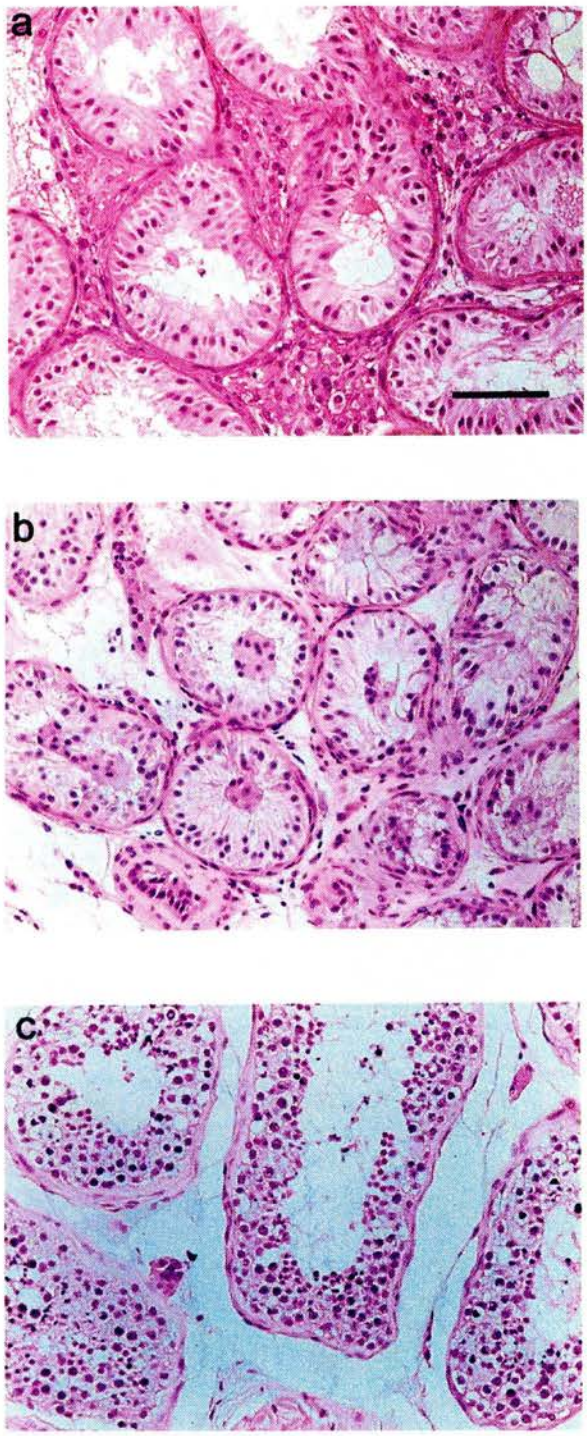

Figure 2. Haemotoxylin and eosin staining: (**a**) and (**b**) show testis morphology pre- and post-hypothalamic–pituitary–gonadal axis suppression respectively, and demonstrate complete absence of all germ cell types, in contrast to the abundant germ cells in the normal seminiferous epithelium of a healthy adult man (**c**). Scale bars = 50 μm.

Semen analysis

All men remained azoospermic throughout the study.

Testicular tissue

Light microscopy

Examination of testicular tissue pre- (Figure 2a) and post- (Figure 2b) HPG axis suppression indicated complete absence of all germ cells, in contrast to the abundant different germ cell types in the normal seminiferous epithelium of a healthy adult man (Figure 2c). This was representative of all seven cancer survivors.

Immunocytochemistry

AR

The nuclei of all cells within the seminiferous epithelium of all patients immunoexpressed AR (Figure 3a, brown staining), and this was comparable before MPA–testosterone treatment and at the end of the study (Figure 3b), in contrast to AR negative cells evident in the testis of a healthy adult man (Figure 3c). This finding suggested that only Sertoli cells were present within the tubules of the cancer patients. Using the methodology applied in the current study, all Sertoli cells in the human testis immunostain for AR with similar intensity (Saunders *et al.*, 1996), although application of modified methods can reveal differences in staining intensity between different Sertoli cells in the normal adult testis (Suarez-Quian *et al.*, 1999).

MAGE-57B antigen

Immunoexpression of MAGE-57B was negative in all specimens, pre- and post-HPG axis suppression, in contrast to the abundant immunopositive germ cells seen in the healthy adult control (Figure 3d–f). This confirms the absence of spermatogonia in the cancer patients.

Discussion

This study demonstrates that suppression of the HPG axis for $\geqslant$3 months, in men rendered azoospermic by gonadotoxic chemotherapy or radiotherapy for childhood cancer, did not result in restoration of spermatogenesis, assessed by both semen analysis and testicular biopsy. In rats, it has been shown that some germ cells survive cytotoxic therapy and that the resulting azoospermia is a consequence of the inability of those spermatogonia that are present to proliferate and differentiate (Kangasniemi *et al.*, 1995). Suppression of the HPG axis facilitates recovery of spermatogenesis following such treatment, and it has been hypothesized that this is the result of a reduction in intratesticular testosterone concentrations (Meistrich and Kangasniemi, 1997). This can be achieved by administration of steroid hormones, or GnRH agonists or antagonists with or without testosterone before, during or after chemotherapy or radiotherapy and such regimens have been demonstrated to enhance recovery of spermatogenesis in rats (Ward *et al.*, 1990; Delic *et al.*, 1986; Pogach *et al.*, 1988; Kangasniemi *et al.*, 1995; Meistrich *et al.*, 1995; Meistrich, 1998). Application of this approach to humans makes the important assumption that the mechanism of cytotoxic chemotherapy or radiotherapy-induced testicular damage is similar in both species, and it has been assumed that like the rat, men might retain a population of spermatogonial stem cells from which spermatogenesis could be regenerated. The mechanism and permanency of impaired spermatogenesis induced by some forms of chemotherapy/radiotherapy in the human may differ more substantially from the rat than was previously appreciated.

The success of hormonal treatment to aid recovery of spermatogenesis in rats subjected to chemotherapy is believed to be based on lowering intratesticular testosterone levels. While the prepubertal testis is relatively quiescent, there is a steady turnover of early germ cells, which undergo spontaneous degeneration before maturation is reached (Muller and Skakkebaek, 1983). This relatively low activity, compared with the adult, does not protect the prepubertal testis from the deleterious impact of cytotoxic therapy, as the present data confirm. The slow turnover of germ cells and their subsequent degeneration in the prepubertal testis may be partly due to low levels of intratesticular testosterone, which is required to complete the end-stages of spermatogenesis (Chemes, 2001). The lack of protection afforded to the prepubertal testis, at a time when testosterone levels are low, would suggest that additional environmental factors play a role in the successful recovery of spermatogenesis in rats and the vulnerability of the prepubertal human testis to cytotoxic therapy. In this regard, our studies with the marmoset, a primate surrogate for man, have demonstrated that activation of testicular cell function occurs well before puberty and is largely gonadotrophin-dependent, but that spermatogonial replication appears to be independent of gonadotrophin stimulation (Kelnar *et al.*, 2002).

In one study in which cyclophosphamide was administered as immunosuppressive therapy for nephrotic syndrome in adult men, preservation of fertility was achieved via supraphysiological testosterone therapy (Masala *et al.*, 1997). Of 15 men treated with cyclophosphamide, five received testosterone to suppress testicular function before and during the 8 month cycle of chemotherapy. All men were azoospermic or severely oligozoospermic within 6 months of commencing cyclophosphamide. Nine of the 10 men who received cyclophosphamide alone remained azoospermic 6 months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all five of the men who received testosterone therapy. High dose cyclophosphamide is known to be associated with impaired spermatogenesis, which is often temporary, and it is probable that in this study the simultaneous administration of testosterone with cyclophosphamide provided some protection or hastened the recovery of spermatogenesis. It would be interesting to have long-term follow-up data on the 10 patients who received cyclophosphamide-only treatment to enable a direct comparison with the natural history of recovery of sperm production. In contrast, other studies have failed to show similar benefits in humans. For example, suppression of testicular function with a GnRH agonist, alone or in combination with testosterone during gonadotoxic chemotherapy treatment for lymphoma, did not confer any protective benefit or enhance recovery of spermatogenesis (Johnson *et al.*, 1985; Waxman *et al.*, 1987). A number of reasons may be considered for the lack of successful outcome in the aforementioned studies. The number of patients and controls studied was small and the cancer therapies variable, in contrast to monotherapy

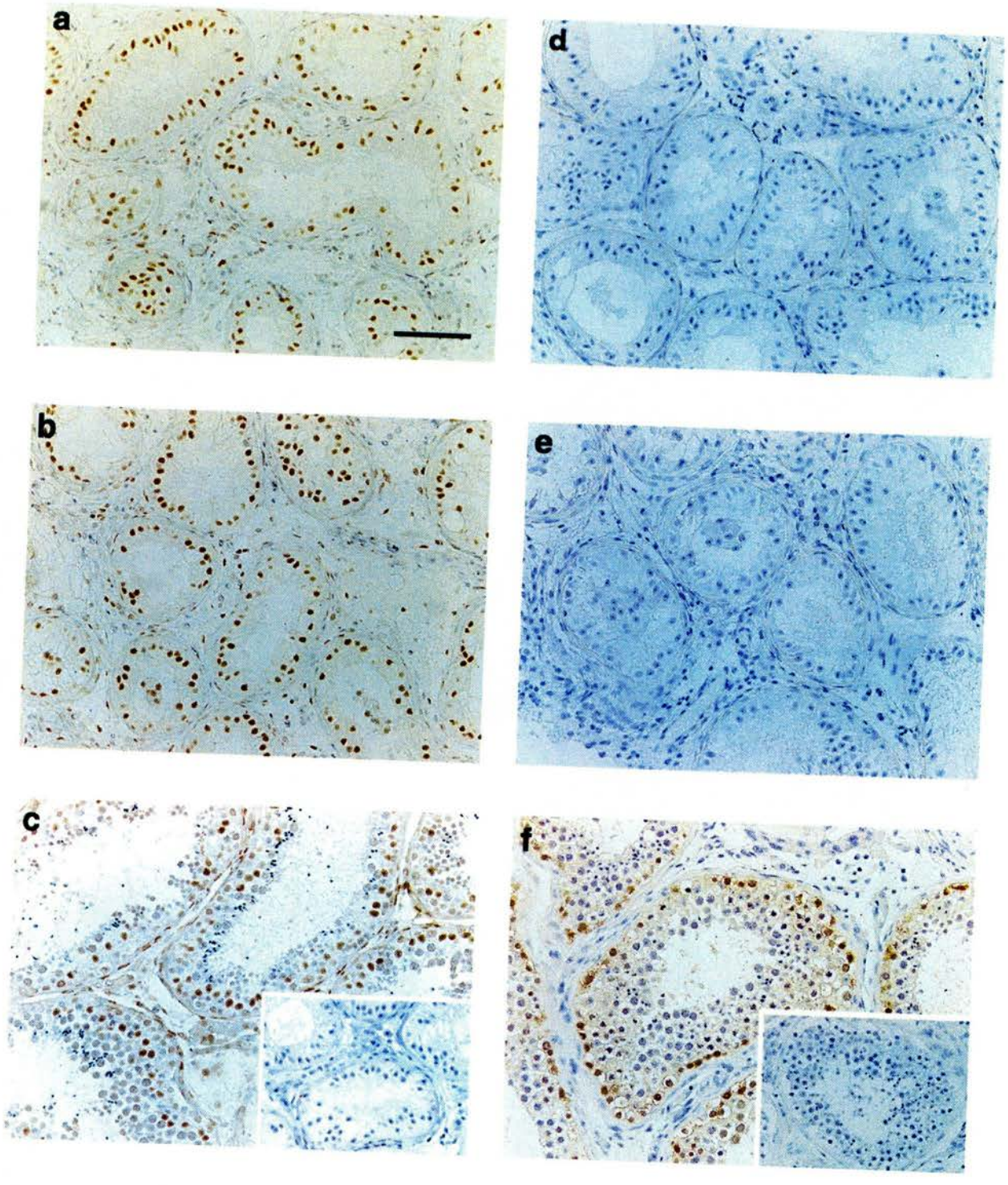

Figure 3. Androgen receptor (AR) staining: (**a**) and (**b**) show AR immunoexpression pre- and post-hypothalamic–pituitary–gonadal axis suppression respectively, and demonstrate that all cell nuclei within the seminiferous tubules are AR immunopositive (brown staining), thus excluding the presence of AR negative germ cells, as evident in the testis of a healthy adult man (**c**). Inset shows negative control in which the primary antibody was omitted. Scale bars = 50 μm. MAGE-57B staining: (**d**) and (**e**) show no evidence of expression of the MAGE-57B antigen (all cells stained blue), both pre-and post-HPG axis suppression, in contrast to the abundant germ cells immunopositive (brown staining) for MAGE-57B demonstrated in the healthy adult control (**f**). Insert shows negative control in which the primary antibody was omitted. Scale bars = 50 μm.

with cyclophosphamide for a non-malignant condition. Treatment regimens may not have been sufficiently gonadotoxic to cause sterility, so no recovery effect could be seen or, conversely, the agents were so gonadotoxic that permanent ablation of all germ cells was induced. Waxman *et al.* studied the protective effects of a GnRH agonist during the treatment of 20 men with cytotoxic chemotherapy for advanced Hodgkin's disease (Waxman *et al.*, 1987). Following administration of the GnRH agonist, standard GnRH testing demonstrated adequate suppression of LH, but not of FSH, throughout the chemotherapy treatment. Follow-up assessment of the men after a 3 year interval showed that all remained severely oligozoospermic (Waxman *et al.*, 1987). In another study, the effect of GnRH agonist administration during combination chemotherapy for advanced lymphoma was evaluated in six patients (Johnson *et al.*, 1985). By 6 years post treatment, only one patient demonstrated any evidence of spermatogenesis. While the present study explored the delayed suppression aspect of this hypothesis, no human studies have combined pre-chemotherapy suppression with continued suppression for a significant length of time following chemotherapy.

A number of steroid hormone combinations have been used to suppress the HPG axis in rats and successfully restore spermatogenesis after chemotherapy, including MPA in combination with testosterone (Velez de la Calle *et al.*, 1990; Jegou *et al.*, 1991). Low dose testosterone, MPA or GnRH analogues alone have been shown to stimulate recovery of spermatogenesis in rats following sterilization with radiotherapy. However, the addition of testosterone to GnRH analogues may reduce the effectiveness of GnRH analogues (Shetty *et al.*, 2000). The combination of testosterone with MPA may also have a reduced effect compared with either agent alone, although this combination results in a profound reduction in intratesticular testosterone concentrations in men (McLachlan *et al.*, 2002). Although further study is warranted, the appropriate choice of hormone suppression will require careful consideration. Long-acting gonadotrophin analogues, such as goserelin, have been shown to be ineffective at suppressing FSH long term in normal men, with recovery of FSH and resumption of spermatogenesis occurring within 2–3 months (Behre *et al.*, 1992; Bhasin *et al.*, 1994).

Inhibin B mediates non-steroidal negative feedback from the testes, reflecting the number of sperm produced and regulating FSH secretion (Andersson *et al.*, 1999; Peterson *et al.*, 1999; Anderson and Sharpe, 2000; Kolb *et al.*, 2000). Inhibin B secretion in the adult requires the presence of germ cells (Andersson *et al.*, 1999). Inhibin B concentrations were barely detectable in the azoospermic patients, despite the preservation of Sertoli cells. This provides further evidence for an essential role of the germ cell–Sertoli cell interaction in the production of inhibin B and confirms the value of inhibin B as a non-invasive marker of spermatogenesis following cytotoxic therapy. Inhibin B was also undetectable in seminal plasma in most subjects, as previously found in men with azoospermia of other aetiologies (Anderson *et al.*, 1998).

Although the gonadotoxic effect of chemotherapy depends upon dosage and drugs administered, and radiotherapy-induced damage upon field of irradiation and dose received, it is difficult to reliably predict the extent of testicular damage and which azoospermic patients may show recovery of spermatogenesis. Our study population comprised an unselected group of seven men rendered azoospermic secondary to treatment for childhood cancer. Testicular biopsies from all seven patients demonstrated complete absence of spermatogonia, yet survival of stem cells is a prerequisite for endocrine restoration of spermatogenesis. It was felt to be unethical to exclude men from the trial on the basis of Sertoli cell-only biopsy specimens for several reasons. Testicular volume in these men was markedly reduced and thus it was justified to take only a small piece of testicular tissue, to eliminate any impact which a reduction in testicular tissue may have on Leydig cell numbers. Small islands of spermatogonia may be present but in our study were absent from the biopsied tissue. Survival of germ cells following apparently sterilizing chemotherapy is evident from a number of studies. Temporary azoospermia and late recovery of spermatogenesis following chemotherapy have been reported, indicating the survival of stem cells (Viviani *et al.*, 1985; Wallace *et al.*, 1991; Pryzant *et al.*, 1993), although permanent azoospermia tends to follow procarbazine and alkylating agent-based regimens, typical of treatment for Hodgkin's disease (Whitehead *et al.*, 1982; da Cuhna *et al.*, 1984; Bramswig *et al.*, 1990). Similar histological findings have been reported in other studies following treatment for Hodgkin's disease with procarbazine-based regimens (Chapman *et al.*, 1979; Charak *et al.*, 1990). With advances in assisted reproduction techniques, the development of testicular sperm extraction (TESE) combined with ICSI offers potential for paternity in these young men. Chan and co-workers report the use of TESE–ICSI to retrieve sperm from men with long-standing azoospermia and achieve a pregnancy (Chan *et al* 2001). Seventeen men, median age (range) 37.4 (28–54) years, had undergone sterilizing chemotherapy treatment 16.3 (6–34) years previously. Of the 17 men, 13 demonstrated Sertoli cell-only on biopsy and the remaining four were described as having hypospermatogenesis. Using microdissection TESE techniques, sperm retrieval was achieved in seven subjects, three (43%) of whom demonstrated Sertoli cell-only on testicular histology and four (57%) with hypospermatogenesis. The seven subjects underwent nine TESE combined with ICSI procedures resulting in a clinical pregnancy in three (33%) and a live birth in two (22%). These encouraging results suggest that microscopic visualization of the seminiferous tubules may enable identification of areas of continued spermatogenesis within the testis and sperm retrieval using microdissection techniques. This reiterates the importance of not excluding men from hormone restoration clinical trials or from assisted reproduction techniques on the basis of a Sertoli cell-only testicular biopsy.

Although small islands of germ cell spermatogonia may exist in the sections of testes that were not biopsied, it is likely that in our patients, the severity of the cytotoxic-induced germ cell loss is such that recovery of spermatogenesis is simply impossible. This does not exclude the possibility that earlier intervention and HPG axis suppression might have been beneficial. However, it seems more probable that HPG axis suppression to restore spermatogenesis may be more successful

in patients in whom the testicular insult is less severe and in whom there is some preservation of spermatogonial stem cells.

Acknowledgements

We gratefully acknowledge the generous support of Pharmacia Corporation and the Child Growth Foundation in funding this study.

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Submitted on November 6, 2001; accepted on March 15, 2002

Best Practice & Research Clinical Endocrinology and Metabolism
Vol. 16, No. 2, pp. 311–334, 2002
doi:10.1053/beem.2002.0200, available online at http://www.idealibrary.com on IDEAL®

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Late reproductive sequelae following treatment of childhood cancer and options for fertility preservation

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The successful treatment of childhood cancer can be associated with impaired gonadal function in adulthood. Chemotherapy and radiotherapy may damage germ-cell spermatogonia, resulting in impaired spermatogenesis or sterility in the male, or may hasten oocyte depletion with truncated fecundity and premature menopause in the female. The only established option in current clinical practice for preserving male fertility is cryopreservation of spermatozoa. The only strategy currently available for preserving female fertility is cryopreservation of embryos. Harvesting and storage of ovarian cortical tissue from girls and young women before potentially gonadotoxic chemotherapy has been available in a number of centres but there have been no live births and the procedure remains experimental. Standards for best practice in the cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures and requirements for safe storage, remain to be defined. Recent advances in assisted reproduction may circumvent natural conception barriers and the implications of impaired DNA integrity may be manifest as an increased risk of congenital abnormalities and chromosomal disorders in the offspring. In this chapter we consider the late reproductive sequelae following treatment for childhood cancer and options for fertility preservation.

Key words: childhood cancer; infertility; cryopreservation; progeny; fertility preservation; cytotoxic gonadal damage; chemotherapy; radiotherapy.

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Continuing therapeutic advances in the management of childhood malignancies mean that the majority of children can realistically hope for long-term survival. With survival rates in excess of 70% it is estimated that by the year 2010 one in 250 of the young adult population will be a long-term survivor of childhood cancer.[1,2] The successful treatment of childhood cancer with chemotherapy and or radiotherapy is associated with significant morbidity in later life.[3] The major challenge faced by paediatric oncologists today is to sustain the excellent survival rates while striving to achieve optimal quality of life.

One of the most frequently encountered and psychologically traumatic late complications of radiotherapy and chemotherapy for childhood cancer is infertility. Consequently, attention is focusing on developing techniques to preserve fertility. Advances in assisted reproduction techniques have focused attention on preserving gonadal tissue for future use.[4–7] For pre-pubertal boys for whom fertility preservation through cryopreservation of semen is not possible, testicular germ cell harvesting provides a technique by which germ cells can be removed and stored during cytotoxic therapy. At a later date, following recovery, the stored tissue could be auto-transplanted or these stored cells could be matured in vitro until they reach a stage sufficiently mature to procure fertilization with assisted reproduction.[7–11] Preservation of female fertility is equally challenging. Embryo cryopreservation is restricted by time constraints, sexual maturity and the presence of a stable partner. Cryopreservation of immature ovarian tissue is the most promising option, with restoration of natural fertility following autotransplantation, or maturation of oocytes in vitro in combination with assisted reproduction.[12,13] Harvesting, and the potential future use of gonadal tissue, is an exciting area of gamete biology which opens up new and uncharted territory for paediatric oncologists managing children with cancer. These techniques are still in the realms of experimental development. A number of ethical and legal issues must be addressed to ensure that their clinical application conforms to a voluntary code of practice, which offers realistic and safe prospects of fertility preservation for young children with cancer.[14]

GONAOAL DAMAGE

Cytotoxic chemotherapy and radiotherapy may damage gonadal tissue and result in long-lasting or permanent sterility. Cancer therapy may damage the developing gonad in children. However, the full impact of the deleterious effects generally remains latent in childhood and is manifest only in adulthood as infertility or a premature menopause.[15–22]

Males

Testis

The testis is highly susceptible to the toxic effects of radiation and chemotherapy at all ages of life. Although the exact mechanism is uncertain it appears to involve a combination of destruction of the proliferating germ cell pool and, where germ cells survive, inhibition of further differentiation.[23] Spermatogenesis is a process, beginning at puberty and continuing throughout adult life, whereby totipotential stem cell spermatogonia undergo continual self-renewal and differentiation into mature spermatozoa.[24] In the relatively quiescent pre-pubertal testis there is a steady

turnover of early germ cells which undergo spontaneous degeneration before the haploid stage is reached; nevertheless, it is likely that it is this steady state that renders the pre-pubertal testis vulnerable to the deleterious impact of cytotoxic therapy.[25–27]

Chemotherapy. Cytotoxic chemotherapy agents may produce long-lasting or permanent damage to the germinal epithelium resulting in oligozoospermia or azoospermia.[15–18,28–34] The extent of the damage is dependent upon the agent administered and the dose received. However, as most treatment is delivered as multi-agent regimens with often synergistic toxicity, it can be difficult to determine the specific contribution of each individual agent. A number have been identified as being gonadotoxic, including alkylating agents (such as chlorambucil and cyclophosphamide), procarbazine, cis-platin and vinblastine (Table 1).[16,17,28–34] Combination chemotherapy treatment of Hodgkin's disease with established regimens (mechlorethamine, vincristine, procarbazine and prednisolone (MOPP) or chlorambucil, vinblastine, procarbazine and prednisolone (ChlVPP) or cyclophosphamide, vincristine, procarbazine and prednisolone (COPP)) have been reported in a number of studies to result in permanent azoospermia in more than 90% of recipients. The gonadotoxic agents in these regimens are mechlorethamine and procarbazine in MOPP, chlorambucil and procarbazine in ChlVPP and procarbazine and cyclophosphamide in COPP.[16,33] The ABVD combination (adriamycin, bleomycin, vinblastine and dacarbazine), which contains neither an alkylating agent nor procarbazine, has been shown to be significantly less gonadotoxic, resulting in temporary azoospermia in 33% of patients and oligozoospermia in 21%, with 'full' recovery after 18 months reported in most, if not all, patients.[33]

'Hybrid' regimens (e.g. alternate cycles of ABVD with cycles of ChlVPP or MOPP) are likely to be less gonadotoxic. Fertility seems to be preserved in approximately 50% of men following three cycles of MOPP/ABVD, in contrast to almost universal azoospermia following six cycles of MOPP.[33]

Cyclophosphamide, either alone or in combination with other agents, is known to damage the germinal epithelium but the threshold for sterility, as with most

Table 1. Gonadotoxic chemotherapy agents.

Alkylating agents
Cyclophisphamide
Ifosfamide
Nitrosoureas, e.g. BCNU, CCNU
Chlorambucil
Melphalan
Busulphan
Vinca alkaloids
Vinblastine
Antimetabolites
Cytarabine
Platinum agents
Cis-platinum
Others
Procarbazine

gonadotoxic agents, is difficult to determine. Follow-up of 30 men for a mean of 12.8 years following treatment with cyclophosphamide (total dose 560–840 mg/kg) for childhood nephrotic syndrome, reported azoospermia in 13%, oligozoospermia in 30% and normal semen analysis in the remaining 57% of the men.[31] The threshold for impaired spermatogenesis was a total dose of 10 g. However, when used for the treatment of solid tumours in combination with doxorubicin and dacarbazine or vincristine, which have not been shown to be gonadotoxic, azoospermia was permanent in 90% of men treated with cyclophosphamide doses >7.5 g/m^2.[35] Management of childhood leukaemia, which is the commonest childhood malignancy, is continually evolving and often includes cyclophosphamide or cytarabine. A study of testicular histology in 44 boys treated for acute lymphoblastic leukemia (ALL) demonstrated a 50% reduction in tubular fertility index (TFI) (percentage of tubules containing identifiable spermatozoa), with severe impairment (TFI $<40\%$) in 18 patients. The severity of the damage was influenced by previous chemotherapy treatment with cyclophosphamide and cytarabine (>1 g/m^2), whereas the tubular fertility index improved with increasing time from treatment.[36] 'Full' recovery of spermatogenesis was observed in three of seven of the patients with severe depression of TFI followed up for median (range), 10.8 (5.5–15.9) years of treatment.[29] Germinal epithelial damage has also been described following successful treatment for childhood ALL with a modified LSA_2L_2 protocol, which includes treatment with cyclophosphamide and cytarabine.[37] In contrast, normal testicular function was reported in 14 boys successfully treated for ALL with treatment which did not include either cyclophosphamide or cytarabine.[38] Current treatment of ALL in the UK includes cytarabine (total dose: 2 g/m^2 or 4 g/m^2) and cyclophosphamide (total dose: 1.2 g/m^2 or 2.4 g/m^2). Although this is unlikely to be sterilizing, long-term follow-up is necessary.

Ifosfamide, an analogue of cyclophosphamide, is another potentially gonadotoxic agent, although limited data describing its effect on testicular function are available. In one study that included five patients treated for Ewing's sarcoma with an ifosfamide-based regimen (total dose: 84–126 g/m^2) only two men had a normal semen analysis, one was oligozoopermic and the remaining two patients were azoospermic.[39]

Radiotherapy. The degree and permanency of radiotherapy-induced testicular damage depends on the treatment field, total dose and fractionation schedule.[15,19,20] Doses as low as 0.1–1.2 Gy damage dividing spermatogonia and disrupt cell morphology, resulting in oligozoospermia.[40,41] Following low-dose, single-fraction irradiation, complete recovery of spermatogenesis was observed 9–18 months following irradiation with 1 Gy, by 30 months following doses of 2–3 Gy and at 5 years or more in those treated with 4 Gy.[40,41] The germinal epithelium appears to be more susceptible to fractionation of radiotherapy, with doses greater than 1.2 Gy fractionated resulting in permanent azoospermia.[20] Leydig cells are more resistant to damage from radiotherapy than is the germinal epithelium and progression through puberty with normal potency is common despite severe impairment of spermatogenesis. Testicular irradiation with doses greater than 20 Gy is associated with Leydig cell dysfunction in pre-pubertal boys while Leydig cell function is usually preserved up to 30 Gy in sexually mature males (Table 2).[42]

In practice, radiotherapy treatment with fractionated total body irradiation (TBI) with 14.4 Gy is liable to render men permanently sterile. Administration of higher doses, for example, 24 Gy for testicular relapse of ALL, is likely to result in sterility and impaired androgen production.

Table 2. Radiotherapy-induced damage to the reproductive tract.

Gender	Site	Effect
Males	Cranial/total body irradiation	Endocrine axis disruption
	TBI/pelvic/testes	Germinal epithelium
		> 1.2 Gy – azoospermia
		0.1–1.1 Gy – oligoozspermia
		Leydig cells
		> 20 Gy – prepubertal
		> 30 Gy – post-pubertal
Females	Cranial/total body irradiation	Endocrine axis disruption
	TBI/abdomen/pelvic	Ovarian failure (LD_{50} < 4 Gy)
		Older women > 5 Gy
		Younger women > 20 Gy
		Uterine damage
		Decreased volume
		Decreased elasticity

Investigation of testicular function. Assessment of testicular maturation and function involves pubertal staging, plasma hormone analysis and semen analysis (Table 3). Pubertal staging provides important clinical information about both Leydig cell function and spermatogenesis.[43] The development of normal secondary sexual characteristics would imply intact Leydig cell function with normal steroidogenesis. Reduced testicular volume (< 15 ml), determined using the Prader Orchidometer, is strongly suggestive of impaired spermatogenesis. Hormone analysis requires measurement of basal plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and inhibin B if available.[44] In pre-pubertal children hormone analysis is an unreliable predictor of gonadal damage because the hypothalamic-pituitary-testicular axis is quiescent. In post-pubertal boys, elevated LH and diminished testosterone levels would indicate Leydig cell dysfunction, while elevated FSH and diminished inhibin B, despite normal plasma testosterone level, would support germ cell failure (Table 2). Semen analysis remains the definitive measure of spermatogenesis.

Inhibin B mediates non-steroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion.[45] Inhibin B secretion in the adult requires the presence of germ cells. This provides further evidence for the essential role of the germ cell–Sertoli cell interaction for the production of inhibin B and confirms the value of inhibin B as a non-invasive marker of spermatogenesis following cytotoxic therapy.

Females

Ovary

Female fertility may be impaired following chemotherapy or radiotherapy treatment for childhood cancer. In contrast to the situation in males, the female gamete pool is fixed at birth, with approximately two million primordial follicles present. This non-renewable pool begins an exponential decline by atresia throughout childhood to adulthood until the number of primordial follicles falls below approximately 1000 at an

average age of 50 years when the menopause occurs. The fertile 'window' in females is characterized by roughly 400 monthly ovulations of a mature oocyte.[46,47] Ovarian failure and a premature menopause will arise from any cytotoxic insult which either depletes the oocyte pool or hastens its decline.[48]

Chemotherapy. Females are generally less susceptible than males to the deleterious effects of chemotherapy on the gonad. Nevertheless, ovarian dysfunction is well recognized following combination chemotherapy. Ovarian damage is drug- and dose-dependent and is related to age at time of treatment, with progressively smaller doses required to produce ovarian failure with increasing age.[49–52] The gonadotoxic agents are similar for males and females (Table 1).

Treatment of Hodgkin's disease with mechlorethamine, vinblastine, procarbazine and prednisolone (MVPP), MOPP or ChlVPP results in ovarian failure in 19–63%.[16,49–52] Amenorrhoea is much more commonly encountered in women over 30 years (50–89%) than in younger women where ovarian function appears to be preserved in 48–100%.[1,49–52] Long-term follow-up will be necessary as a number of these young women may subsequently progress to a premature menopause.[48]

Treatment for childhood ALL appears to be associated with preservation of ovarian function in the majority of cases.[53–55] In a UK study of 40 girls treated for childhood ALL, all achieved adult pubertal development and 37 had regular menses. There were 14 live births in nine long-term survivors with no serious congenital abnormalities and no reported cases of malignant disease in their offspring.[56] A recent study has shown decreased LH excretion and short luteal phase in young women treated for ALL in childhood. This subtle ovulation disorder is probably related to low-dose cranial irradiation.[57]

Ovarian failure following high-dose chemotherapy treatment as conditioning for bone marrow transplantation appears to be temporary. In a study of 43 women with aplastic anaemia, cyclophosphamide conditioning (200 mg/kg) resulted in amenorrhoea in all women, with 36 showing recovery of normal ovarian function 3–42 months after transplantation.[58] Ovarian function was normal in 95% of females treated pre-pubertally with cyclophosphamide (200 mg/kg) as conditioning therapy for bone marrow transplantation. However, doses in excess of 200 mg/kg may result in

Table 3. Assessment of gonadal function.

Testicular dysfunction	
Leydig cell dysfunction	Reduced testosterone
	Elevated LH
Germinal epithelial	Reduced testicular volume
	Elevated FSH
	Low inhibin B
	Impaired spermatogenesis
Ovarian dysfunction	
Ovarian failure prepubertally	Delayed puberty
	Primary amenorrhoea
Ovarian failure during or post-puberty	Arrested puberty
	Secondary amenorrhoea
	Menopausal symptoms
Endocrine profile	Elevated FSH
	Reduced oestradiol

premature ovarian failure.[59] Studies are currently underway to evaluate the impact of ifosfamide, an analogue of cyclophosphamide, on ovarian function. Patients receiving combination chemotherapy within continually evolving protocols require vigilant long-term follow-up to monitor the risk of late effects.[3]

Radiotherapy. Total body, abdominal or pelvic irradiation may cause ovarian and uterine damage. The degree of impairment is related to radiation dose, fractionation schedule and age at time of treatment.[21,22,56,59] The number of primordial follicles present at the time of treatment and dose of radiotherapy will determine the fertile 'window' and dictate the age at menopause. This means that the younger the child at the time of radiotherapy the larger the oocyte pool and hence the later the menopause. The human oocyte is sensitive to radiation, with an estimated LD_{50} of less than 4 Gy.[22] A permanent menopause may be induced in women over 40 years following treatment with 6 Gy while significantly higher doses are required to destroy the oocyte pool completely and to induce ovarian failure in younger women and children.[19,21]

Ovarian failure has been observed in 97% (37 of 38) of females following whole abdominal irradiation in childhood (20–30 Gy). Of the 37 women, primary amenorrhoea was reported in 71% and premature menopause (median age 23.5 years) in the remainder.[21]

Total body irradiation (TBI), either alone or in combination with cyclophosphamide (as conditioning for bone marrow transplantation), is associated with infertility. All women conditioned with total body irradiation (9.2–15.75 Gy) and cyclophosphamide (120 mg/kg) before bone marrow transplantation for leukaemia develop amenorrhoea, with recovery seen in only nine of 144. TBI was found to be the only factor significantly influencing ovarian failure – the younger the age of the patient at the time of treatment the greater the probability of recovery of ovarian function.[58] In a long-term follow-up of 708 women, median 3 (range: 1–17) years, after bone marrow transplantation, 532 had received TBI (10–15.75 Gy, single exposure or fractionated) and 176 were treated with cyclophosphamide (200 mg/kg), alone or with busulphan (16 mg/kg), as conditioning therapy. Ovarian failure was observed in 90% of patients following TBI and 68% following

Table 4. Clinical and experimental strategies for the preservation of reproductive function in oncological patients.

Clinical practice	
Males	*Females*
Sperm banking	Oophoropexy
Ejaculation	
Rectal electro stimulation	
Testicular/epididymel aspiration	
	Embryo cryopreservation
Experimental strategies	
Males	*Females*
Cryoperservation of immature spermatogenic cells	Cryopreservation of oocytes
	Gonadotrophin suppression
Gonadotrophin suppression	Inhibition of follicle apoptosis
Cryopreservation of testicular tissue	Cryopreservation of ovarian tissue

chemotherapy among an additional 82 patients treated pre-pubertally with the same regimens, ovarian failure was reported in 72%.[60]

Investigation of ovarian function. Clinical manifestations of ovarian damage depend upon the age of the child at the time of treatment (Table 3). Cytotoxic damage sustained pre-pubertally may result in failure to develop secondary sexual characteristics. If the child is peri-pubertal at the time of treatment there may be arrest of pubertal development with subsequent primary amenorrhoea. In post-pubertal females ovarian failure will manifest as secondary amenorrhoea, often with symptoms of oestrogen deficiency. Biochemically, ovarian failure is detected as elevated gonadotrophins, and undetectable oestradiol. Prediction of a premature menopause is difficult, although early follicular phase assay of follicle-stimulating hormone (FSH), oestradiol and inhibin B and ovarian ultrasound are potential tools for assessing ovarian reserve. Elevated early follicular phase FSH with preservation of oestradiol production is characteristic of the peri-menopause, the length of which may vary.[61,62] Ovarian failure will require oestrogen replacement for pubertal induction and cyclical hormone replacement to relieve the symptoms of oestrogen deficiency (vaginal dryness, hot flushes and irritability), provide cardiovascular protection and optimize bone density.

Uterus

Radiotherapy and chemotherapy. Recent data demonstrate that uterine function may be compromised following radiotherapy. Reduced uterine volume and decreased elasticity of uterine musculature, possibly as a consequence of impaired vascularization, are found in girls receiving pelvic, abdominal and total body irradiation pre-pubertally.[63,64] Disruption of uterine characteristics may compromise implantation and continuation of pregnancy. Although successful pregnancies following radiotherapy are reported, the incidence of spontaneous miscarriage, premature delivery and intrauterine growth retardation is significantly increased.[21,60,65–67] Following whole abdominal irradiation (20–30 Gy) in childhood, all pregnancies occurring in women with preserved ovarian function resulted in mid-trimester miscarriage.[21] In studies exploring the role of exogenous sex steroids, women with premature ovarian failure following TBI for childhood leukaemia, treated with physiological sex steroid replacement therapy, have shown an increase in uterine volume and endometrial thickness.[64,68] This is encouraging for future fertility prospects and may be a useful way of improving uterine function when assessing these women for assisted reproductive techniques.

Chemotherapy does not appear to have any significant lasting adverse effect on uterine function. Successful pregnancy, with no increased risk of miscarriage, and healthy offspring are reported following treatment with multi-agent chemotherapy regimens.[37,69,70]

Investigation of uterine function. Normal uterine shape, length and volume for pre-, peri- and post-puberty are well documented.[71] Ultrasound scanning is a reliable non-invasive technique for assessing uterine size and shape, blood supply and endometrial thickness. Uterine artery blood flow may be assessed by Doppler scanning and resistance to flow expressed as the pulsatility index. Endometrial biopsy enables assessment of endometrial function using histological and immunocytochemistry techniques.[72,73]

FERTILITY PRESERVATION

Advances in assisted reproduction and increasing interest in gamete extraction and maturation have focused attention on preserving gonadal tissue from children before sterilizing chemotherapy or radiotherapy, with the realistic expectation that future technologies will be able to utilize their immature gametes. The impetus for preserving gonadal tissue follows on the heels of pioneering experiments in ewes,[74] together with media interest in the report of a successful autologous ovarian graft in a previously oophorectomized female. In addition, live human births have been reported resulting from the transfer of embryos fertilized with immature spermatogenic cells.[75–80] Such issues have inevitably raised questions from parents and oncologists about their possible application in children undergoing cancer therapies.[14,81]

Males

Potential strategies for preservation of male fertility are dependent upon the sexual maturity of the patient (Table 4). Spermatogenesis is a highly organized and complex process in which undifferentiated diploid stem cell spermatogonia undergo a series of proliferation, differentiation and maturation into mature haploid spermatozoa. This highly efficient process begins in puberty, although a number of immature spermatogenic cells are described in the pre-pubertal testis.[24] Spermarche is a mid-pubertal event, preceding the ability to produce an ejaculate, which occurs at a median age (range) of 13.4 (11.7–15.2) years when median testicular volume is 11.5 (4.7–19.6) ml.[82]

Established practice

Cryopreservation of gametes. The only established current clinical option for preservation of male fertility is cryopreservation of spermatozoa. Spermatozoa are usually obtained from the ejaculate by masturbation but may be obtained using rectal electrostimulation techniques under anaesthetic. When it is not possible to obtain an ejaculate, sperm can be retrieved by epididymal aspiration or testicular biopsy in sexually mature men. Not infrequently, sperm produced by cancer patients at the time of diagnosis is of poor quality. With advances in assisted reproduction techniques, in particular intracytoplasmic sperm injection (ICSI), which involves the injection of a single spermatozoan directly into an ooctye, the problems of low numbers and poor motility sperm may be circumvented.[5–7] More recently a small number of pregnancies have been achieved with ICSI using immature spermatids and secondary spermatocytes extracted from testicular tissue in men with spermatogenic arrest.[75–80]

Experimental strategies

Gonadotrophin suppression. Azoospermia following cytotoxic cancer therapy was believed to be a consequence of destruction of the rapidly proliferating spermatogonial stem cells. In man this would result in azoospermia within 12 weeks, the time taken from differentiation of stem cell to mature spermatozoa, yet spontaneous recovery of spermatogenesis is reported after many years of azoospermia.[40,41] Furthermore, the relatively quiescent pre-pubertal testis is not afforded any protection from cytotoxic therapy. To address these incongruities, studies in rats have shown that some stem

cells survive cytotoxic therapy and that ensuing infertility is a consequence of the inability of spermatogonial stem cells to differentiate.[23] Failure of spermatogonial stem cells to differentiate is likely to result from loss of appropriate environmental endocrine or paracrine growth factors. Studies have shown that administration of gonadotrophin-releasing hormone (GnRH) agonists or antagonist to rats, either immediately or after a delay following treatment with sterilizing irradiation or chemotherapy, restores the ability of spermatogonia to differentiate and resume normal spermatogenesis. Similar results have been achieved using testosterone, alone or in combination with oestradiol or progestin. Despite the attractiveness of this theory studies in humans have failed to demonstrate any convincing evidence of clinical benefit.[83,84] In men treated with sterilizing radiotherapy and chemotherapy for childhood cancer, effective gonadotrophin suppression with medroxyprogesterone acetate for at least 3 months did not result in restoration of spermatogenesis.[85] Studies in the marmoset, a primate animal model, have demonstrated that the pre-pubertal testis is not quiescent and that both GnRH-dependent and -independent activity occurs in pre-puberty.[27] Survival of stem cells is a prerequisite for endocrine enhancement of recovery of spermatogenesis. The absence of histological evidence of spermatogonial stem cells in testicular biopsies from these men demonstrated that there may have been complete ablation of the germinal epithelium and infertility was likely to be irreversible. Endocrine manipulation to enhance recovery of spermatogenesis may be successful in patients in whom the testicular insult is less severe and there is some preservation of spermatogonial stem cells.

Harvesting testicular tissue. For pre-pubertal boys, lacking in haploid gametes, there are no options currently available to preserve fertility and any potential strategies must be considered entirely experimental. In theory, testicular tissue could be removed before the start of treatment and cryopreserved either as a segment of tissue or as isolated germ cells. Following cure from his malignancy the frozen thawed testicular tissue could be auto-grafted to the testes or undergo enzymatic digestion to isolate the germ cells. Frozen thawed germ cells could be re-implanted back into his own testes, or these cells could be matured in vitro until they reach a stage sufficiently mature to achieve fertilization with intra-cytoplasmic sperm injection (ICSI).

The concept of testicular germ cell transplantation was pioneered by Brinster and collegues in 1994.[9] Testicular germ cells isolated from mouse testis and transplanted into the testis of genetically or experimentally sterile mice initiated and sustained normal donor spermatogenesis, restoring fertility and producing healthy offspring.[85] Successful transplantation has also been shown in mouse recipients rendered sterile with the chemotherapeutic agent busulfan. Interestingly, these experiments also demonstrated both endogenous and donor spermatogenesis simultaneously, indicating that busulfan did not kill all the endogenous stem cells.[9] Subsequent studies have shown that testicular germ cell transplantation can occur through autologous, heterologous (mouse-to-mouse, rat-to-rat) or xenologous (hamster, dog, rabbit or rat to mouse) transfer of cells. The site can be heterotopic, as graft to a non-gonadal site in the recipient, or homotopic, when the cells are re-introduced into the testis.[9–11,86–88] In order to develop autologous germ cell transplantation in humans techniques have to be developed by which human testicular germ cells can be isolated, stored and re-introduced into the testis.

Cryopreservation of whole tissue. Testicular tissue obtained by testicular biopsy has been successfully cryopreserved with subsequent isolation of spermatozoa from the thawed tissue and successfully used in ICSI.[89,90] These studies have only utilized spermatozoa or immature spermatogenic cells retrieved from tissue post-thaw and it may be that survival of spermatogonial germ cells post-thaw is poor. Furthermore, as there is a more than theoretical risk of transplanting tumour cells, it is likely that clinical practice will involve enzymatic digestion and isolation of germ cells before cryopreservation rather than autografting testicular tissue.

Germ cell isolation and storage. Transplantation in rodent models has involved only semi-purified cell populations of germ cells and somatic cells but for human application purified stem cell populations would be necessary to ensure that no malignant cells were transferred. The term 'stem cell' is a functional description, given that there are no morphological, antigenic or biochemical criteria by which to identify these cells in vivo or in vitro. Spermatogonial transplantation is the only method at present by which stem cell presence can be authenticated. In mice LacZ encodes the enzyme β-galactosidase which can be demonstrated histochemically as an intracellular blue reaction product. Effective purification will demand the development of specific antibody probes to differentiate stem cells from other cells. Studies have shown that a number of cell surface antigens are expressed on stem cells, including alpha-6, beta-1 integrin and c-kit, which may enable enrichment using magnetic cell sorting.[91] The major limitation for human application is that these antigens are shared with other progenitor cells, including haematopoietic cells, creating inherent problems in cancer patients.

Germ cell enrichment. The highly efficient process of spermatogenesis begins in puberty, although a number of immature spermatogenic cells are described in the pre-pubertal testis. It is believed that 10^4 germ cells contain as few as two stem cell spermatogonia capable of self-renewal.[26] With the average number of germ cells in the testis estimated to range between 13 and 83×10^6 during childhood, the relatively small numbers of stem cell spermatogonia available pose a challenge for the process of stem cell isolation.[25] Consequently, there is a need to develop an in vitro culture system to augment stem cell numbers following harvesting and isolation. The feasibility of enrichment has been shown by Nagano et al who have maintained mouse stem cells in culture for up to 4 months and the cultured cells initiated successful spermatogenesis following transplantation.[92]

Cryopreservaton of spermatogonial stem cells. Cryopreservation of spermatozoa and fertilization following thawing are well established. Application of this approach to stem cell spermatogonia will require modification and optimization of freezing procedures, taking into consideration the inherent biological differences between the immature diploid stem cells and mature gametes.[93] There is thus a need for studies to devise cryopreservation regimens that are applicable to stem cells from the pre-pubertal testis.

Future use of germ cells. The future use of testicular germ cells is likely to involve either maturation of the germ cells in vitro for use with ICSI or transplantation. Ideally, transplantation would involve injecting preparations of purified germ cells into

the testis with restoration of natural fertility. In vitro maturation techniques, although still very much in their infancy, would have the advantage of eliminating any risk of transplanting harvested malignant cells back into the patient.

In vitro maturation. Creating an environment in vitro to simulate germ cell maturation and differentiation into spermatozoa may be the only option for a number of patients where cancer therapy damage has been so extensive that the supporting Sertoli cells would be unable to support spermatogenesis. Attempts to cultivate male germ cells in vitro have shown that germ cells can survive several months in culture and are likely to be undergoing cell division.[92] Tesarik reported in vitro spermatogenesis and healthy offspring using ICSI. However, the maturation process involved in vitro maturation of late stages of spermatogenesis rather than development from germ cells.[94,95]

Germ cell transplantation. A number of studies have concentrated on developing the most efficient technique for infusing the stem cells into the testis. The simplest and most effective method of filling the seminiferous tubules in vitro proved to be by injection into the rete testis of bull, monkey and man, under ultrasound guidance.[96] These experiments involved injection into partially involuted or pre-pubertal testes. This eliminates the problems associated with back pressure of fluid in fully active testes, which would otherwise block the infusion of the cell suspension into the tubules. Successful in vivo rete injections have been performed on cynomologus monkeys treated with GnRH antagonist. The advantage of injecting the cells into the rete allows a much larger volume to be infused in contrast to the micro-injections involved in re-infusing directly into the seminiferous tubules.[96] Further work is required to perfect this technique for human application.

Xenogeneic grafting of testicular tissue. Immature testicular tissue has been shown to grow and differentiate when grafted into another species.[10] This provides an additional strategy for conserving the male germ line and circumvents the risk of re-introducing malignant cells. Clearly, this technique is unlikely to be ethically acceptable and is compromised by the risk of interspecies transfer of potentially pathogenic micro-organisms.

Females

As with males, the options available are dependent upon the sexual maturation of the patient, although in contrast to males, the gamete pool is fixed at birth and collection is technically more difficult. A number of strategies for protecting the ovaries and preserving fertility during cancer therapy have been attempted with limited success (Table 4). Limitation of radiation dose to the ovary is practised but is not very effective. In young, sexually mature females with partners, collection of mature oocytes for storage or fertilization and subsequent embryo cryopreservation is possible. For pre-pubertal girls, and the majority of young women, preservation of fertility remains experimental and harvesting and storage of gonadal tissue before commencing cancer therapy is the most promising option.

Established practices

Surgical translocation. Reducing the radiation dose to the ovary by shielding or removing the ovaries from the field of radiation (oophoropexy) may preserve ovarian function.[96–99] Oophoropexy involves laparoscopic transposition of the ovaries (with blood supply intact) to a position behind the uterus, which acts as a shield, or to the paracolic gutters, away from the field of radiation to minimize exposure. Experimentally, heterotopic ovarian autotransplantation has involved grafting the ovary to a distant site in the body and anastomosing blood vessels.[98] The ovarian dose received during pelvic nodal irradiation for Hodgkin's disease can be limited by midline oophoropexy from 44 Gy to between 0.22 and 0.55 Gy, and in women who are less than 25 years of age at the time of treatment, ovarian failure is infrequent.[97] However, even where ovarian function is preserved oocyte retrieval with assisted reproduction and surrogacy may be required to achieve a pregnancy as the uterus may also have been damaged by the radiation therapy. This will compromise to ability of the women to carry a pregnancy to term.

Storage of embryos or mature oocytes. The only strategy currently available for preservation of female fertility is cryopreservation of embryos. Embryo cryopreservation and in vitro fertilization (IVF) may be offered to women before treatment for cancer. Embryo banking has the advantage that it allows a number of embryos to be stored without a need for further IVF cycles; however, this requires the patient to have a partner or to use donor sperm. On average 10 oocytes are collected per cycle, limiting the number of embryos available for cryopreservation. The overall live birth rate from a single cycle of treatment is 11%.[100] Cryopreservation of oocytes is an alternative possibility for women without a partner but is much less successful, with fewer than one baby born per 100 oocytes stored and as such remains experimental.[101] The main disadvantage of embryo or oocyte storage is requirement for superovulation with gonadotrophins which will inevitably delay the commencement of cancer therapy.

Experimental strategies

Gonadotrophin suppression. The mechanism of cytotoxic chemotherapy and radiotherapy-induced gonadal damage is uncertain and may differ in males and females, between different modalities of therapy and individual drugs. In contrast to males, females appear to be less susceptible to the cytotoxic effects of chemotherapy. Consequently it was hypothesized that inducing a pre-pubertal milieu during chemotherapy would decrease the risk of premature ovarian failure. Gonadotrophin analogues (GnRH-a) prevent follicular growth and mitosis by blocking gonadotrophin induction. Although the exact mechanism is uncertain it may involve direct suppression of GnRH receptors in the ovary, with subsequent inhibition of recruitment of small follicles into the proliferating pool as well as atresia of the already developed follicles. A number of studies have demonstrated that GnRH-a inhibit chemotherapy-induced ovarian follicular depletion in rodents, although there remains uncertainty about applicability in the human, particularly as the human ovary has significantly fewer numbers of GnRH receptors in the ovary.[102–104] Ataya et al demonstrated that GnRH-a co-treatment protected the Rhesus monkey from cyclophosphamide induced ovarian damage by significantly reducing follicular decline compared with cyclophosphamide alone.[103] These findings are supported by clinical studies which demonstrated that co-treatment of GnRH-a

with chemotherapy resulted in premature ovarian failure (POF) in one out of 28 (3.6%) compared with 26 out of 40 (65%) in the group treated with chemotherapy alone.[104] Adjuvant treatment with GnRH-a to limit the gonadal toxic effects of otherwise successful treatment regimens is potentially attractive. However, this must be viewed with some caution as although GnRH-a provided some protection against cyclophosphamide no advantage was conferred against irradiation-induced damage. This may be in part explained by the different mechanism of gonadal damage induced by radiotherapy, namely, the destruction of primordial follicles which are not under the influence of gonadotrophins.[105] The judicious use of GnRH-a may play a role in the appropriate patient group, such as young women and children subjected to alkylating-agent-based chemotherapy for Hodgkin's disease.

Prevention of follicle atresia. Oocyte loss induced by anticancer therapy has been shown to occur by apoptosis; consequently, inhibition of the apoptotic pathway has been explored as a mechanism for preventing ovarian failure. Although the exact pathway remains to be elucidated, accumulating data support the role of ceramide in signalling somatic cell death. Ceramide is a sphingolipid second messenger derived from sphingomyelinase-catalysed hydrolysis, in addition to de novo synthesis. In turn, ceramide is metabolized and phosphorylated to give sphingosine-1-phosphate (S1P) which is believed to inhibit apoptosis in somatic cells. The role of the sphingomyelin pathway has also been studied in germ cells. Disruption of the gene encoding acid sphingomyelinase or treatment with sphingosine-1-phosphate attenuates apoptosis of primordial fetal oocytes with increased oocyte numbers present at birth. Treatment of mice ooctyes with sphingosine-1-phosphate prevents chemotherapy-induced apoptosis in vitro. In vivo administration of sphingosine-1-phosphate confers resistance to radiation-induced apoptosis in mice, with pregnancy rates of 100%.[106] While S1P may herald promise of a new approach to preservation of ovarian function, further studies are necessary to explore the detrimental effects of such treatment on normal neurological function as deletion of sphingomyelinase during normal fetal life leads to the development of Niemann–Pick-disease-like symptoms in post-fetal life.[106]

Harvesting ovarian tissue. Oocyte maturation and storage is possible in post-pubertal girls but is associated with limited success.[100] The only option potentially available for pre-pubertal children and the majority of young women is the cryopreservation of ovarian tissue. Harvesting ovarian tissue may take the form of cryopreservation of slices of ovarian cortex, which are rich in primordial follicles, or cryopreservation of immature oocytes. Good survival rates and viability post-thawing is similar whether primordial follicles are stored as slices or in isolation, in contrast to immature oocytes, where survival is poor and increased rates of chromosomal abnormalities and spindle malformation are reported.[12,107]

Future use of ovarian tissue

Ovarian tissue transplantation. The harvesting of ovarian tissue, either whole ovary or cortical strips, can readily be achieved laparoscopically before potentially sterilizing cytotoxic therapy. The tissue can be safely cryopreserved until required for future use. A potential scenario would be to replant the ovarian tissue with the hope of restoring natural fertility and also maintaining sex steroid production. Autologous transplantation of fresh and frozen-thawed primordial follicles to the ovaries of sterile recipients

has restored fertility, resulting in live healthy offspring in mice and sheep.[74,108–110] Human primordial follicles survive cryopreservation, and the return of ovarian hormonal activity has been achieved with re-implantation. However, no pregnancies have yet been reported and this procedure must be considered experimental.[111,112] It is likely that ovarian grafts will have a limited life span, in which case transplantation should be delayed until fertility is desired.

In vitro culture. Alternative strategies are required because of concerns that the re-implanted ovarian tissue may transmit the original malignant disease. This is of particular concern for haematological malignancies. Fresh and frozen-thawed ovarian tissue from diseased mice transmitted lymphoma when transplanted into healthy recipients.[113] This highlights the risk of potential transmission of cancer cells and emphasizes the need to ensure that adequate procedures are developed to enable storage of tumour-free tissue and the careful selection of the appropriate patients from whom to harvest tissue. In view of the potential risk of transplanting tumour cells a number of alternative strategies are being investigated.

The risk of transplanting tumour cells can be eliminated by maturing the ovarian follicles in vitro followed by assisted reproduction.[111,114] In vitro culture of secondary pre-antral follicles to antral stages using FSH stimulation has been successful in a number of species, including mouse, hamster, pig and humans.[115–120] Maturation of secondary mouse follicles in vitro with fertilization of the oocytes has produced healthy offspring.[116] Despite this, secondary follicles are few in number in human ovaries and development into early antral stages is associated with a very high rate of oocyte loss.[119] Consequently, successful and realistic in vitro maturation of human tissues dictates the development of techniques to mature the much more abundant, primordial follicle.[120,121] At present this is limited by our current lack of knowledge of the signals involved in inducing primordial follicle growth into secondary follicles, although it is presumably mediated by interaction with surrounding stromal cells.[119] Culture of primordial follicles may be approached by culture of ovarian cortical slices, which would maintain the structural integrity and enable interaction between follicles and surrounding stromal cells. Alternatively, primordial follicles could be isolated and cultured, which would have the advantage of allowing direct monitoring of follicular development. Culture of primary and primordial follicles to pre-ovulatory and pre-antral stages, respectively, has been achieved in mice, with subsequent isolation and maturation of the secondary follicles and a live offspring produced following IVF in one case.[122] Cultures from enzymatically isolated primary and primordial follicles from mice, rats and pigs have yielded similar degrees of development.[122–125] Isolated fresh and frozen-thawed human primordial follicles have been successfully maintained in culture for up to 5 days but no evidence of growth was observed.[12]

Xenogeneic transplantation. Heterotopic xenogeneic transplantation is one alternative. Studies have shown that the renal capsule of immunodeficient mice can successfully serve as a site for transplantation of ovarian slices from various species.[126,127] Ovarian cortical slices from sheep, cats and marmosets have been transplanted to immunodeficient mice and developed to late antral stages, providing a model and a host for follicular development.[128,129] Similar studies transplanting human ovarian slices to SCID mice have produced similar results following administration of follicle-stimulating hormone.[130]

Clinical practice for harvesting gonadal tissue

Harvesting and storage of ovarian cortical tissue from girls and young women before gonadotoxic chemotherapy has been available in a number of centres since the mid-1990s and, more recently, a few centres have reported the storage of testicular tissue.[131] The Royal College of Obstetricians and Gynaecologists has provided a report from a working party on the storage of ovarian and pre-pubertal testicular tissue. This provides standards for best practice in the cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures and requirements for safe storage.[132]

PROGENY

Overall, there are reassuring reports that there is no increased incidence of either congenital abnormalities or childhood malignancy in children born to long-term survivors of childhood cancer.[133,134] However, these successful pregnancies result mostly from normally achieved conception. We do not know the consequences of circumventing the natural selection processes of normal sexual reproduction using assisted reproduction techniques (ART), nor the effects of ART on the complex cascade of precisely timed molecular interactions of early embryonic development. Continued surveillance of the progeny of survivors of childhood cancer remains essential.[135]

Paternal risk to the offspring

The mutagenic potential of cancer therapy may confer a risk to the fetus conceived using gametes produced after cancer therapy, although current epidemiological data suggest that offspring of cancer survivors do not have an increased incidence of congenital abnormalities or cancer relative to the general population.[133,135] However, an important concern is that these results are based largely on offspring arising from natural conception and the consequences of circumventing the natural selection processes of fertilization involved by means of ICSI are unknown.[135] There is at least the hypothetical possibility of injection of abnormal spermatozoa or immature spermatogenic cells carrying abnormal genomic DNA with the potential to increase congenital and other abnormalities among offspring.[136] Studies in animals have shown that exposure of the male germ line to chemotherapy agents may disrupt spermatozoal DNA and result in deleterious effects on embryo development.[137–139] Awareness of the importance of sperm DNA integrity for accurate transmission of genetic material to the offspring has necessitated the development of new techniques to assess sperm characteristics in more detail.[137] It has become clear that men from subfertility clinic populations, with abnormalities of the conventional criteria of semen quality, also demonstrate elevated levels of damage to the genomic DNA in their gametes. Even among normal populations, sperm chromatin damage has been linked with impaired fecundity.[140] It has been shown that sperm DNA damage does not preclude pronucleus formation at ICSI, and that abnormal DNA within the male gamete is detectable in the early embryo.[141] Thus far, evidence on the safety of ICSI has been based largely upon its use in populations of men with deficits in spermatogenesis unrelated to potentially mutagenic cancer treatment. This evidence has been broadly reassuring concerning health risks to the offspring, although it is limited

by the restricted length of follow-up currently available.[142] Studies have shown that although, by conventional criteria, semen quality is frequently abnormal in long-term survivors of childhood cancer, the sperm produced do not appear to carry a greater burden of damaged DNA.[39] This observation goes some way to providing reassurance about the use of ICSI, which will circumvent the problems associated with severe oligozoospermia and asthenozoospermia, and offer cancer survivors the possibility of paternity in adulthood.

As assisted reproduction techniques advance, successful pregnancies are achieved with immature spermatogenic cells, adding a further unquantified risk to the fetus. Fertilization of oocytes with immature spermatogenic cells, such as round cells and elongated spermatids, which have not yet completed spermatogenesis, must be pursued with caution. The mechanism by which sperm precursor cells activate the oocyte at fertilization is uncertain but it is speculated that suboptimal oocyte activation may confer poor fertilization, implantation and high early abortion rates.[143] Spermatid transition into spermatozoa is characterized by salient changes in nuclear protein composition; the significance of circumventing these changes is uncertain.[144] Genetic imprinting is reported to play an important role in embryogenesis and in processes leading to the development of paediatric cancers, including Wilms' tumour and embryonal rhabdomyosarcoma, and other human diseases.[145] Although the mechanism involved in genetic imprinting is uncertain it is likely to involve differences in DNA methylation and requires careful consideration when embarking on germ-cell maturation. Children born following assisted conception using spermatozoa and immature spermatogenic cells require long-term careful monitoring.

Maternal risk to the offspring

As with males there is the theoretical risk that exposure to chemotherapeutic agents or irradiation may cause mutations and DNA changes to the oocyte. Animal studies have demonstrated high abortion and malformation rates related to different stages of oocyte maturation at the time of exposure to chemotherapy.[60,70,146] This has raised concerns regarding the use of assisted reproduction techniques and embryo cryopreservation in patients previously exposed to cancer therapy. Reassuringly, studies of pregnancy outcome in cancer survivors have not substantiated these concerns. There is no increased incidence of chromosomal or congenital abnormalities in offspring born to women exposed to cancer therapy.[60,70,146]

CONCLUSIONS

As treatment for childhood cancer has become increasingly successful, adverse effects on reproductive function are assuming greater importance. Preservation of fertility before treatment must be considered in all young patients at high risk of subfertility. Limitation of radiation exposure by shielding of the testes and ovaries should be practised where possible and sperm banking should be offered to all sexually mature boys at risk of infertility. The rapidly advancing experimental techniques for harvesting of gonadal tissue must be considered and embarked upon without unrealistic expectations, although future utilization of the tissue is likely to be realized in the next decade.

Semen can be stored before sterilizing chemotherapy and radiotherapy for use at a later date, thus preserving fertility. Attempts to restore spermatogenesis in azoospermic

men, using hormone manipulation, have so far been unsuccessful. However, capitalizing on the unique properties of the spermatogonial stem cell, testicular tissue could be harvested before treatment and cryopreserved, for use at a later date. The functional capacity of the testes appears to be preserved and autotransplantation of the stem cells in rats has been shown to re-initiate spermatogenesis and permanently restore fertility. Where fears of re-introducing tumour cells may limit applicability of this approach in humans, in vitro maturation and use with assisted reproduction techniques is a realistic alternative.

Girls undergoing treatment for childhood cancer face limited options for preservation of fertility. Harvesting ovarian tissue and cryopreservation of cortical slices are practised in a number of centres although these procedures remain experimental and future utilization of stored tissue is uncertain. Ultimately, autografting and restoration of natural fertility is the aim of cryopreservation of ovarian tissue but techniques need to be developed to ensure that malignant cells are not transferred in the tissue. Isolation of follicles and in vitro maturation for assisted reproduction is likely to be the best option and may be the only option when the uterus has also been damaged. It is likely that these techniques will be developed in the not too distant future and preservation of fertility will be a realistic expectation for girls treated with gonadotoxic therapy.

It is incumbent upon oncologists that appropriate counselling of patients at risk of subfertility be part of their routine care. The importance of the late effects of treatment are receiving increasing recognition and in the UK strategies for long-term follow-up are currently being explored.[3]

Practice points

- successful treatment of childhood cancer may be associated with impaired gonadal function in adulthood
- the testis is highly susceptible to the toxic effects of radiation and chemotherapy at all ages of life
- assessment of testicular maturation and function involves pubertal staging, plasma hormone analysis and semen analysis
- females are generally less susceptible than males to the deleterious effects of chemotherapy
- total body, abdominal or pelvic irradiation may cause ovarian and uterine damage and the degree of impairment is related to the radiation dose, fractionation schedule and age at time of treatment
- apart from cryopreservation of spermatozoa in the male and embryos for the female, all alternative strategies remain experimental
- human primordial follicles survive cryopreservation, and return of ovarian hormonal activity has been achieved with re-implantation; however, no pregnancies have yet been reported and this procedure must be considered experimental
- best practice in the cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures and requirements for safe storage need to be established
- concerns that the offspring of patients successfully treated for cancer might have an increased risk of congenital abnormalities and childhood cancer have not been substantiated

Research agenda

Males
- isolation, identification and storage of stem spermatogonia
- in vitro maturation of stem spermatogonia
- in vivo transplantation of testicular tissue

Females
- harvesting of ovarian tissue
- in vitro maturation of primordial follicles
- autologous transplantation of ovarian tissue
- prevention of follicle atresia by novel stategies

General
- a voluntary code of practice for collection of gonadal material
- safe cryopreservation techniques need to be developed
- epidemiological surveillance of offspring of survivors of childhood cancer after ART

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Clinical Endocrinology (2003) **58**, 296–301

Is inhibin B a potential marker of gonadotoxicity in prepubertal children treated for cancer?

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(Received 12 July 2002; returned for revision 28 August 2002; finally revised 4 September 2002; accepted 27 September 2002)

Summary

BACKGROUND AND OBJECTIVE **Chemotherapy treatment of childhood cancer may impair gonadal function, which may be manifested only in adulthood as permanent sterility. Detection of gonadal dysfunction in prepubertal children has been hampered by the absence of a sensitive marker. Inhibin B is secreted by small antral follicles and Sertoli cells in females and males, respectively, and may be a marker of gonadal function in prepubertal children. The aim of this pilot study was to evaluate inhibin B in relation to sensitive measurements of gonadotrophins as markers of the early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer.**

STUDY DESIGN AND SUBJECTS **Twenty-five prepubertal children (9 females), median age 4·5 years (range 1·2–12·8 years) with cancer (16 solid tumours, nine acute lymphoblastic leukaemia, ALL) were studied longitudinally. Blood samples were collected before and during chemotherapy (solid tumours) or immediately following induction and first intensification (ALL). Post-treatment (1–6 months) samples were collected in 12 of the patients (5 females).**

MEASUREMENTS **Dimeric inhibin B was measured by double antibody enzyme-linked immunosorbent assay (ELISA). FSH and LH were measured by sensitive time-resolved immunofluorescence.**

RESULTS ***Girls*: Pretreatment inhibin B was slightly high in one girl but normal for age and sex in all others: median 16·1 (range 9·4–186·2) ng/l, median SD score +0·2 (–1·3 to +2·6). Inhibin B decreased to undetectable levels (< 8 ng/l) in 8/9 girls during treatment ($P = 0·03$), with no accompanying rise in FSH or LH. Post-treatment recovery of inhibin B was variable: median 16·1 (range < 8·0–44·2) ng/l, median SD score +0·1 (range < –2·4 to +1·8). Sustained undetectable inhibin B levels were observed in 2/5 girls with correspondingly elevated FSH concentrations (11·8 and 10·9 U/l).**

***Boys*: Inhibin B was normal for age and sex in all boys before treatment with no significant change during or after treatment (medians 93 ng/l, 85 ng/l and 94 ng/l, SD scores –0·3, –0·6 and –0·2, respectively). Inhibin B decreased to undetectable levels in one boy post-treatment with no accompanying increase in FSH or LH.**

CONCLUSIONS **In prepubertal girls with cancer, chemotherapy is associated with suppression of inhibin B, usually transient, which may indicate arrest of follicle development. Sustained suppression of inhibin B following treatment may be indicative of permanent ovarian damage. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed effect. Inhibin B, together with sensitive measurements of FSH, may be a potential marker of the gonadotoxic effects of chemotherapy in prepubertal children with cancer.**

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The successful treatment of childhood cancer may be associated with gonadal dysfunction and infertility in adulthood (Waring & Wallace, 2000; Thomson *et al.*, 2002). Chemotherapy and radiotherapy may damage testicular germinal epithelium resulting in temporary or permanent azoospermia in the male, or hasten oocyte depletion with truncated fecundity and a premature menopause in the female. Although chemotherapy may damage the gonads in both sexes throughout life, females appear to be less susceptible to the deleterious effects of chemotherapy than males. The extent of chemotherapy-induced damage is dependent upon the agent administered and dose received. However, it can be difficult to determine the relative contribution of each individual

drug as most treatments are administered as multiagent regimens. A number of agents have been identified as being gonadotoxic, including cisplatin, melphalan and the alkylating agents, such as cyclophosphamide.

Traditionally, determining the impact of chemotherapy and radiotherapy on gonadal function has involved clinical assessment of pubertal development, menstrual history in females and semen analysis in males. Earlier detection of gonadal damage has been hampered by the lack of a sensitive marker of gonadal function in prepubertal children. In these children gonadotrophins may be unreliable predictors of gonadal damage because the hypothalamic–pituitary–gonadal axis is relatively quiescent. Inhibin B is a dimeric glycoprotein produced by the gonads, secreted from Sertoli cells in males and developing antral follicles in females (Roberts *et al.*, 1993; Anderson & Sharpe, 2000). In the adult male inhibin B plays an important role in spermatogenesis, while in the adult female it plays an integral role in folliculogenesis, oocyte maturation and corpus luteal function, mediated in part by inhibition of FSH secretion. There is increasing evidence reporting that the prepubertal gonads are not entirely quiescent but have a low level of activity (Wu *et al.*, 1996; Kelnar *et al.*, 2002). We investigate the role of inhibin B, together with sensitive gonadotrophin measurements, as markers of early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer.

Patients and methods

Patients

Stored plasma was analysed from blood samples collected for previous prospective, longitudinal studies exploring the effects of chemotherapy on growth and bone turnover (Crofton *et al.*, 1998; Bath *et al.*, in press). The original studies and their extension to this study were approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee. The previous studies involved sequential recruitment of 47 children (18 females), median age 4·5 years (range 1·2–15·3 years), presenting with a variety of haematological and solid malignancies over a 2-year period (Crofton *et al.*, 1998; Bath *et al.*, in press). From this cohort we excluded postpubertal patients ($n = 7$), germ cell tumours ($n = 1$) and patients for whom there was insufficient plasma stored before or during treatment ($n = 14$). The study group consisted of 25 clinically prepubertal children (nine girls). The median age (range) at diagnosis was 4·5 (1·2–12·8) years and underlying malignancies included acute lymphoblastic leukaemia (ALL, $n = 9$, females 3), neuroblastoma ($n = 5$, females 1), non-Hodgkin's lymphoma ($n = 3$, females 0), rhabdomyosarcoma ($n = 3$, females 2), osteosarcoma ($n = 2$, females 1), Ewing's sarcoma ($n = 1$, females 1), medulloblastoma ($n = 1$, females 1) and Wilms ($n = 1$, females 0). Children with solid tumours ($n = 16$) were treated with multiagent chemotherapy regimens in accordance with United Kingdom Childrens Cancer Study Group (UKCCSG) protocols. One female also received lumbar radiotherapy for Ewing's sarcoma (L3). Children with ALL ($n = 9$) were treated with the MRC UKALL XI protocol. We collected blood samples from patients with solid malignancies ($n = 16$) before and during chemotherapy (median number of cycles 7, range 4–14) and from patients with ALL ($n = 9$) pretreatment and at the end of week 6 (after completion of induction and first intensification). In 12 of the 25 children treated for cancer, follow-up blood samples were collected between 1 and 6 months after completion of chemotherapy.

Methods

Blood samples were separated immediately after collection and plasma stored at −70°C until analysis. Dimeric inhibin B was measured in duplicate by a double antibody enzyme-linked immunosorbent assay (ELISA), using a monoclonal antibody raised against a synthetic peptide from the βB subunit, combined with an antibody to an inhibin α subunit sequence, as described (Groome *et al.*, 1996; Crofton *et al.*, 2002a). The assay detection limit was 8 ng/l. Within- and between-assay coefficients of variation (CVs) were 9·6% and 13·0% at 19 ng/l, 7·4% and 10·6% at 88 ng/l, and 8·4% and 10·2% at 233 ng/l, respectively. FSH and LH were measured in duplicate by Delfia time-resolved immunofluorescence assays (Wallac, Milton Keynes, UK), standardized against the second International Reference Preparations of pituitary FSH and LH, coded 78/549 and 80/552, respectively. Assay detection limits were 0·3 U/l for FSH and 0·2 U/l for LH. Between-assay CVs for FSH were 6·5% at 5·2 U/l, 3·5% at 10·4 U/l and 3·6% at 35·3 U/l, and for LH were 5·9% at 6·4 U/l, 5·5% at 11·3 U/l and 4·4% at 18·7 U/l.

Data analysis

Inhibin B varies with age and sex. Data are presented both as absolute concentrations (ng/l) and as SD scores calculated for age and sex following log-transformation, based on our own published data (Crofton *et al.*, 2002a,b). Group results are presented as medians and ranges. Within-individual changes through time were evaluated by Wilcoxon matched pairs.

Results

Girls

Inhibin B, FSH and LH results in the children before, during and after chemotherapy are shown in Table 1. Pretreatment inhibin B levels were comparable to the normal age- and sex-matched

Table 1 Median (range) of inhibin B, FSH and LH levels in children before, during and after chemotherapy

	Before	During	After
Girls			
*N**	9 (6)	9 (6)	5 (5)
Inhibin B (ng/l)	16·1 (9·4, 186·2)	< 8·0 (< 8·0, 20·8)†	16·1 (< 8·0, 44·2)
Inhibin B (SDS)	+0·2 (− 1·3, +2·6)	−‡	+ 0·1 (< −2·4, +1·8)
FSH (U/l)	0·7 (< 0·3, 1·9)	1·0 (< 0·3, 3·0)	6·0 (1·4, 11·8)
LH (U/l)	< 0·2 (< 0·2, 0·2)	< 0·2 (< 0·2, < 0·2)	< 0·2 (< 0·2, 1·1)
Boys			
*N**	16 (10)	16 (10)	7 (6)
Inhibin B (ng/l)	93 (77, 244)	85 (58, 261)	94 (< 8, 170)
Inhibin B (SDS)	−0·3 (− 1·9, +1·8)	−0·62 (−1·8, +1·8)	−0·18 (< −7·0, +1·4)
FSH (U/l)	< 0·3 (< 0·3, 1·2)	< 0·3 (< 0·3, 0·8)	0·4 (< 0·3, 2·6)
LH (U/l)	< 0·2 (< 0·2, 0·3)	< 0·2 (< 0·2, < 0·2)	< 0·2 (< 0·2, 0·3)

*Total number of children with cancer (number with solid tumours).
†$P < 0·05$ compared with pretreatment levels.
‡Not reported because 8/9 girls had undetectable inhibin B during chemotherapy (see text).
SDS, SD score according to age and sex.

population in eight of the nine girls. One clinically prepubertal girl, aged 10·5 years, had a high inhibin B level for age (SD score +2·6) but prepubertal gonadotrophin levels (FSH 1·9 U/l, LH 0·2 U/l). All other girls had pretreatment inhibin B levels within their respective age- and sex-specific reference ranges and appropriate for prepubertal girls (Crofton *et al.*, 2002a). FSH and LH concentrations were prepubertal in all nine girls.

During treatment, inhibin B decreased to undetectable levels in eight of the nine girls ($P = 0·03$ compared with pretreatment samples) (Fig. 1a) with no accompanying rise in FSH or LH (Table 1). The only girl in whom inhibin B was not suppressed was treated with low doses of potentially gonadotoxic agents (cyclophosphamide 1·5 g/m^2, cisplatin 80 mg/m^2 and carboplatin 1·0 g/m^2).

Samples were available from five girls after completion of treatment. Analysis of post-treatment samples demonstrated variable recovery of inhibin B, with levels remaining below the assay detection limit in two girls (Fig. 1a). FSH (but not LH) increased in all girls post-treatment compared with their pretreatment levels ($P = 0·06$, Table 1). The two girls (aged 2·6 and 10·5 years, respectively) in whom inhibin B remained undetectable post-treatment had the highest post-treatment levels of FSH (11·8 and 10·9 U/l, respectively).

Boys

Inhibin B levels were appropriate for age with no significant changes before, during and after treatment in all but one boy (Table 1). FSH and LH levels remained prepubertal in all patients pre-, during and post-treatment (Table 1). Following completion of treatment, six of the seven boys showed little change in inhibin B (Fig. 1b). In one patient (aged 2 years), inhibin B levels decreased to undetectable levels following completion of treatment with alkylating agent-based gonadotoxic chemotherapy (cyclophosphamide, 4·4 g/m^2, cisplatin 320 mg/m^2 and melphalan 200 mg/m^2) with no post-treatment increase in gonadotrophins (FSH 0·8 U/l, LH < 0·2 U/l).

Discussion

Girls

Chemotherapy treatment of childhood cancer is associated with suppression of inhibin B during treatment, which may indicate arrest of follicular development. Recovery of ovarian function following completion of chemotherapy treatment is variable and is likely to reflect the severity of the gonadotoxic insult. Some of the girls treated with known gonadotoxic agents demonstrated sustained suppression of inhibin B levels and continued assessment will be necessary to observe if this is transient or associated with impaired ovarian function and development of a premature menopause.

At diagnosis, prepubertal girls' inhibin B levels were appropriate for age and sex, indicating that they continued to maintain normal ovarian follicular development, at least to the small antral stage. During treatment with a variety of combination chemotherapy regimens, inhibin B was suppressed in all but one girl, indicating arrest of follicular development from the primordial pool. The exception was a girl in whom chemotherapy had been curtailed owing to lack of tumour response and who had therefore been exposed to relatively low doses of potentially gonadotoxic chemotherapy. There was no accompanying increase in FSH levels in the girls with suppressed inhibin B, consistent with hypothalamic-pituitary quiescence during chemotherapy in these prepubertal girls. Although all three girls with ALL had undetectable inhibin B at the end of first intensification (which

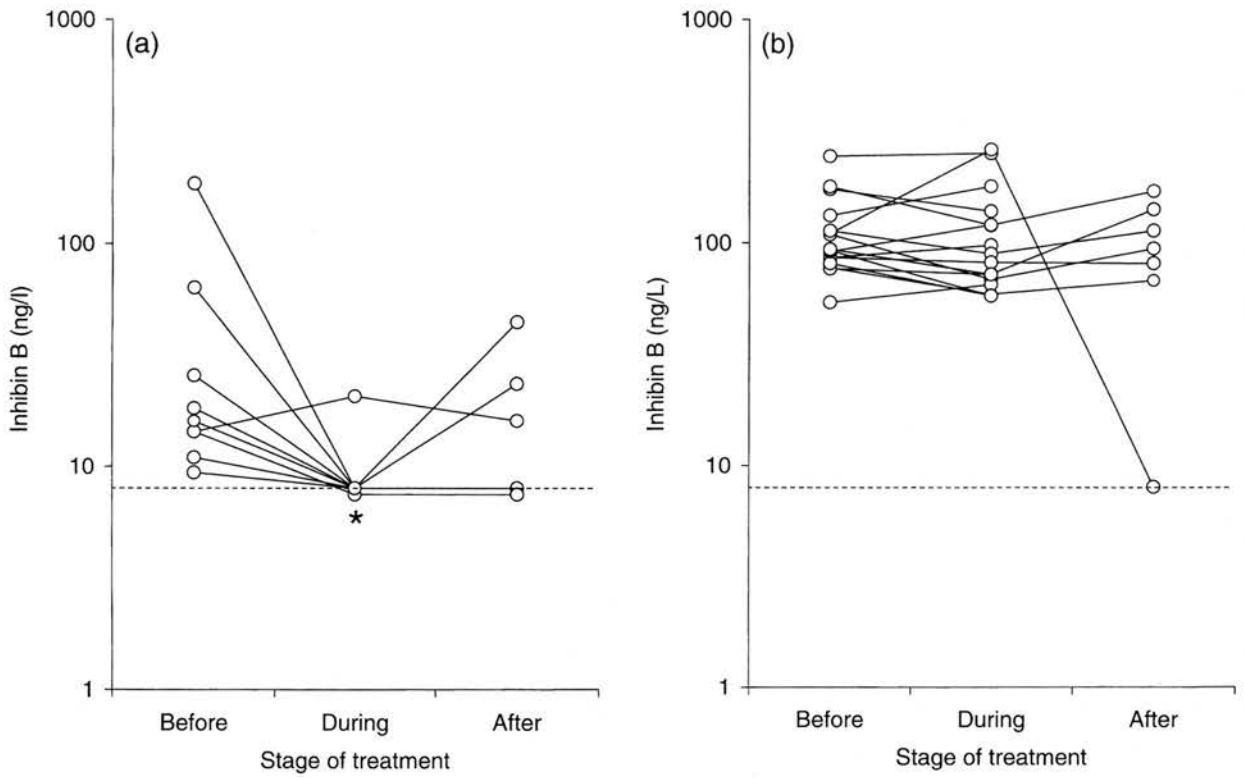

Fig. 1 Inhibin B levels in individual children with cancer before, during and after treatment: (a) girls; (b) boys. Inhibin B is plotted on a log scale. The dotted line represents the detection limit of the inhibin B assay. Results below the assay detection limit are plotted at the detection limit. $*P < 0{\cdot}05$ compared with pretreatment levels (Wilcoxon matched pairs). See text for details.

includes cytarabine, a potentially gonadotoxic agent), inhibin B returned to normal, detectable levels 2–4 weeks later and remained normal in all three girls during the second year of continuing chemotherapy (data not shown). These results suggest that arrest of folliculogenesis in girls treated for ALL is likely to be transient. This is consistent with retrospective studies of survivors of childhood ALL that have reported relatively normal ovarian function and reproductive pattern in females treated with chemotherapy (Nygaard *et al.*, 1991; Wallace *et al.*, 1993).

After completion of treatment, there was variable recovery of inhibin B. Two girls in whom inhibin B remained undetectable had elevated post-treatment levels of FSH, suggesting resumption of pituitary activity with loss of feedback suppression on FSH production. One of these girls was aged 10·5 years at the start of treatment and 11·3 years old at the time of follow-up, 6 months after completion of treatment, an age at which there is normally active follicular development to the small antral stage with detectable inhibin B in all girls (Crofton *et al.*, 2002a). This girl therefore appeared to have persistent defective folliculogenesis following gonadotoxic chemotherapy (cisplatin 600 mg/m^2). Cisplatin doses of this magnitude have been shown to be associated with long-term ovarian damage in survivors (Wallace *et al.*, 1989). Post-treatment inhibin B was also undetectable in a 2-year-old patient treated for neuroblastoma with combination chemotherapy including the gonadotoxic agents cisplatin (320 mg/m^2), cyclophosphamide (4·2 g/m^2), and melphalan (200 mg/m^2) as preconditioning therapy for autologous bone marrow transplantation. At this age inhibin B may be undetectable on random sampling in up to 35% of normal girls (Crofton *et al.*, 2002a). However, her FSH increased from 0·7 U/l pretreatment to 11·8 U/l post-treatment. The combination of undetectable inhibin B and increased FSH is suggestive of ovarian damage.

Although the numbers in our study are small, the data suggest that the arrest of follicular development associated with potentially gonadotoxic chemotherapy may persist in some girls during the early post-treatment period. In other girls, inhibin B and FSH levels were normal after completion of treatment, with a positive relationship between the two hormones, as observed in normal prepubertal girls (Crofton *et al.*, 2002a). This is consistent with resumption of follicular development under the influence of FSH. Long-term follow-up of all patients is warranted to monitor for the development of premature ovarian failure.

Boys

Inhibin B, produced from Sertoli cells, mediates nonsteroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion (Pierik *et al.*, 1998). Inhibin B secretion in the adult requires the presence of germ cells and in the prepubertal boy may reflect continuous Sertoli cell proliferation and functional activity together with maturation of early germ cells and spontaneous degradation. Men with complete absence of germ cells (Sertoli cell only syndrome, SCOS), severe hypospermatogenesis and spermatogonial and/or spermacytic arrest have lower inhibin B levels and higher FSH levels than normozoospermic controls (Mahmoud *et al.*, 1998; Bohring & Krause, 1999; Foresta *et al.*, 1999). Gonadotoxic chemotherapy in men is associated with a decrease in circulating inhibin B during treatment (Wallace *et al.*, 1997). We have recently demonstrated reduced sperm concentration, reduced inhibin B and increased FSH levels in male survivors of childhood cancer (Thomson *et al.*, 2002). Chemotherapy has been implicated as the cause of these endocrine abnormalities in adult survivors of childhood cancer (Lähteenmäki *et al.*, 1999; Schmiegelow *et al.*, 2001). However, a longitudinal study of prepubertal boys with acquired SCOS caused by irradiation/chemotherapy for ALL has demonstrated that reductions in inhibin B levels are generally delayed until postpuberty (Andersson *et al.*, 1998).

In our study, boys with cancer had normal inhibin B levels before treatment started and these changed little during and after chemotherapy except for one boy with neuroblastoma treated with relatively high doses of known gonadotoxic agents, cyclophosphamide, cisplatin and melphalan (Watson *et al.*, 1985; Jaffe *et al.*, 1988; Aubier *et al.*, 1989; Wallace *et al.*, 1989; Chatterjee *et al.*, 1994; Waring & Wallace, 2000) in whom inhibin B became undetectable following completion of treatment, suggestive of Sertoli cell damage. This may reflect damage to the germinal epithelium and long-term follow-up and monitoring of testicular function will be essential. Our results show that inhibin B levels were normal in boys with ALL throughout treatment, including year 2 of continuing chemotherapy (data not shown), consistent with reports of normal testicular function and normal spermatogenesis in most male survivors of childhood ALL (Blatt *et al.*, 1981; Wallace *et al.*, 1991). FSH and LH remained low or undetectable in all prepubertal boys with cancer before, during and after treatment, illustrating their insensitivity as markers of gonadal damage before the onset of puberty. In the boy who developed undetectable inhibin B, there was no accompanying increase in FSH, consistent with previous evidence that inhibin B and FSH secretion are independent in prepubertal boys, in contrast to the inverse relationship that develops later during puberty (Crofton *et al.*, 2002b).

In summary, prepubertal girls treated with chemotherapy developed suppressed inhibin B levels indicating arrest of follicular development, with variable short-term recovery of folliculogenesis after completion of treatment. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed deleterious effect. This pilot study has not addressed the long-term implications for future fertility. Further studies are planned, combining inhibin B with FSH, LH and sex hormone measurements, to assess longer term reversibility and delayed effects, particularly as the children approach and progress through puberty.

Acknowledgements

We are grateful to the Jennifer Fund for their generous support of this study.

Dr S. F. Ahmed was involved in the collection of samples from the patients with ALL. We also thank Dr A. Thomas at the Royal Hospital for Sick Children, Edinburgh, for allowing her patients to be studied.

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REVIEW

Preservation of fertility in children treated for cancer

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Arch Dis Child 2003;**88**:493–496

As treatment for childhood cancer has become increasingly successful, adverse effects on reproductive function are assuming greater importance. Preservation of fertility before treatment must be considered in all young patients at high risk of infertility, and provision of such services requires collaboration between oncology centres and assisted conception units. The UK Children's Cancer Study Group is planning to audit current management of preservation of reproductive function in young patients with cancer, and the British Fertility Society is preparing a voluntary code of best practice to guide and inform clinicians and scientists. Limitation of radiation exposure by shielding of the testes and ovaries should be practiced where possible and sperm banking should be offered to all sexually mature boys at risk of infertility. The rapidly advancing experimental techniques for harvesting of gonadal tissue must be considered and embarked on without unrealistic expectations, although future utilisation of the tissue is unlikely to be realised until the next decade.

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Accepted 5 October 2002

Improvements in the diagnosis, management, and treatment of childhood cancers have made the prospect of survival into adulthood a realistic expectation for the majority of children. However, it is a recognised complication that certain forms of treatment may compromise fertility.[1 2] Consideration of fertility preservation is a quality of life issue at a time of intense stress for young patients and their families. Nevertheless, in our experience, open discussion is embraced and often potentially therapeutic for the vulnerable family facing treatment for cancer. Discussion of fertility issues at the time of diagnosis provides the family with the reassurance that the oncology team believe in a future when these issues will become important.

Treatment of childhood cancer with chemotherapy and radiotherapy may damage gonadal tissue and result in permanent sterility in both males and females.[3–18]

RADIOTHERAPY INDUCED GONADAL DAMAGE

The nature of radiotherapy induced gonadal damage depends on the field of treatment, total dose, and fractionation schedule.[3–5] In males, doses as low as 0.1–1.2 Gy can damage dividing spermatogonia and disrupt cell morphology, resulting in oligozoospermia.[3 4] Permanent azoospermia has been reported following single fraction irradiation with 4 Gy or 1.2 Gy fractionated.[3 4] Leydig cells are more resistant to damage from radiotherapy than the germinal epithelium, and progression through puberty with normal potency is frequent, despite severe impairment of spermatogenesis. Testicular irradiation with doses of greater than 20 Gy is associated with Leydig cell dysfunction in prepubertal boys, while Leydig cell function is usually preserved up to 30 Gy in sexually mature males.[5]

Total body, abdominal, or pelvic irradiation may cause ovarian and uterine damage.[6–9] The human oocyte is sensitive to radiation, with an estimated LD_{50} of less than 2 Gy.[6] The younger the child at the time of radiotherapy the larger the number of primordial follicles present, hence for a given radiation exposure the longer the "window" of fertility before a premature menopause ensues. Ovarian failure will require oestrogen replacement for pubertal induction and cyclical hormone replacement to relieve the symptoms of oestrogen deficiency (vaginal dryness, hot flushes, and irritability), provide cardiovascular protection, and optimise bone density. However, even where ovarian function is preserved, this does not guarantee fecundity as radiation damage to the uterus may have occurred. Uterine irradiation in childhood increases the incidence of nulliparity, spontaneous miscarriage, and intrauterine growth retardation.[8 9] The mechanism underlying these findings remains unclear, but reduced elasticity of the uterine musculature and uterine vascular damage have been suggested.[8 9]

CHEMOTHERAPY INDUCED GONADAL DAMAGE

The impact of combination cytotoxic chemotherapy on gonadal function is dependent on gender and age of the child undergoing treatment, and the nature and dosage of the drugs received.[9–18] Drugs known to cause gonadal damage include procarbazine, cytosine arabinoside, and the alkylating agents, particularly cyclophosphamide, chlorambucil, mustine, melphalan, busulphan, and the nitrosoureas.[9–18] Treatment for Hodgkin's disease in the UK with "ChlVPP" (chlorambucil, vinblastine, procarbazine, prednisolone) is known to cause gonadal damage, particularly in the male, and the agents implicated are chlorambucil and procarbazine. In a recent long term follow up study, 89% of the males treated before puberty had evidence of

Abbreviations: GnRH, gonadotrophin releasing hormone; HFEA, Human Fertilisation and Embryology Authority; ICSI, intracytoplasmic sperm injection; S1P, sphingosine-1-phosphate

severe damage to the germinal epithelium and recovery of spermatogenesis would be unlikely.[16] Around 50% of girls treated for Hodgkin's disease prepubertally with six or more courses of ChlVPP had raised plasma gonadotrophin concentrations, but longer follow up is needed to determine whether these women have recovery of function or go on to develop a premature menopause.[16] The use of ABVD (Adriamycin, bleomycin, vinblastine, dacarbazine), which does not contain alkylating agents or procarbazine, is significantly less gonadotoxic.[17] Current regimens with hybrid protocols are likely to preserve fertility in women and in approximately 50% of men.

Chemotherapy does not appear to have any significant lasting adverse effect on uterine function. Successful pregnancy, with no increased risk of miscarriage, and healthy offspring have been reported following treatment with multiagent chemotherapy regimens.[18]

PRESERVATION OF FERTILITY

Current practice

Potential strategies for preservation of fertility are dependent on the sexual maturity of the patient. The only established option for preservation of male fertility is cryopreservation of spermatozoa.[19] Spermatozoa are usually obtained from the ejaculate by masturbation. When it is not possible to obtain an ejaculate, sperm can be retrieved by epididymal aspiration or testicular biopsy in sexually mature males. Not infrequently, sperm produced by cancer patients at the time of diagnosis is of poor quality. However, with advances in assisted reproduction techniques, in particular intracytoplasmic sperm injection (ICSI), which involves the injection of a single spermatozoon directly into an oocyte, the problems of low numbers and poor motility sperm may be circumvented.[20] In our view any spermatozoa retrieved, either from the ejaculate or surgically, should be cryopreserved irrespective of the perceived quality of the material. Data on the health of offspring born after ICSI are broadly reassuring.[21] A recent study showed that although the conventional criteria of semen quality are frequently abnormal in long term survivors of childhood cancer, the sperm produced do not appear to carry a greater burden of damaged DNA. This observation goes some way to providing reassurance about the use of ICSI, which will circumvent the problems associated with severe oligozoospermia and offer cancer survivors the possibility of paternity in adulthood.[22]

A number of strategies to protect the ovaries and preserve fertility during cancer therapy have been attempted with limited success. In contrast to males, the gamete pool in females is fixed at birth and collection is technically more difficult. In young sexually mature females with partners, collection of mature oocytes for fertilisation and subsequent embryo cryopreservation is possible.[23] Cryopreservation of oocytes is an alternative possibility for women without a partner but is less successful. On average 5–10 oocytes may be harvested per patient with fewer than one baby born per 100 oocytes stored.[24] The main disadvantage of embryo or oocyte storage is the requirement for superovulation with gonadotrophins, which will inevitably delay the commencement of cancer therapy. Limitation of radiation dose to the ovary is sometimes practiced in adult women, but in children is technically difficult.[25]

Experimental strategies

For prepubertal children, lacking in haploid gametes, there are no options currently available to preserve fertility, and any potential strategies must be considered entirely experimental. One approach to preserving fertile potential was based on the original idea that the prepubertal gonads are quiescent and therefore protected from the cytotoxic effects of chemotherapy and radiotherapy which destroy rapidly dividing cells. It was hypothesised that suppression of the hypothalamic-pituitary-gonadal axis by administration of gonadotrophin releasing hormone (GnRH) analogues would render the gonads less susceptible. While it has since become clear that the prepubertal gonads are susceptible to the deleterious effects of cytotoxic therapy, there is significant evidence for the success of protection/recovery strategies in rats. However, clinical studies in man have to date been inconclusive.[26 27] In men treated with sterilising radiotherapy and chemotherapy for childhood cancer, effective gonadotrophin suppression with medroxyprogesterone acetate for at least three months did not result in restoration of spermatogenesis.[27] A number of studies have shown that GnRH analogues inhibit chemotherapy induced ovarian follicular depletion in rodents by blocking gonadotrophin induction.[28] These findings are supported by clinical studies which showed that co-treatment of GnRH analogues and chemotherapy resulted in primary ovarian failure in 1 of 44 (2.3%) compared with 26 of 45 (58%) in the group treated with chemotherapy (with or without mantle field irradiation) only.[28] The judicious use of GnRH analogues may play a role in the appropriate patient group, such as young women and children subjected to alkylating agent based chemotherapy for Hodgkin's disease.

Oocyte loss induced by cytotoxic therapy has been shown to occur by apoptosis; consequently inhibition of the apoptotic pathway has been explored as a mechanism for preventing ovarian failure. Sphingosine-1-phosphate (S1P), a metabolite of ceramide, is believed to inhibit apoptosis in somatic cells. Treatment of mice ooctyes with S1P prevents chemotherapy induced apoptosis in vitro. In vivo administration of S1P confers resistance to radiation induced apoptosis in mice, with pregnancy rates of 100%.[29] While S1P may herald promise of a new approach to preservation of ovarian function, further studies are necessary to explore the detrimental effects of such treatment on normal neurological function, as deletion of sphingomyelinase during normal fetal life leads to the development of Neimann-Pick disease-like symptoms in post-fetal life.

Advances in assisted reproduction and increasing interest in gamete extraction and maturation have focused attention on preserving gonadal tissue from children before sterilising chemotherapy or radiotherapy, with the realistic expectation that future technologies will be able to utilise their immature gametes. The impetus for preserving gonadal tissue follows on the heels of pioneering experiments in ewes,[30] together with media interest in the report of an autologous ovarian graft in a previously oophorectomised female with return of, albeit short lived, menstrual cycle.[31] In addition, live human births resulting from the transfer of embryos fertilised with immature spermatogenic cells have been reported.[32] Such issues have inevitably raised questions from parents and oncologists about their possible application in children undergoing treatment for cancer.[33 34]

In theory gonadal tissue could be removed before the start of treatment and cryopreserved, either as a segment of tissue or as isolated germ cells. Following cure from his/her malignancy the frozen thawed gonadal tissue could be used in a number of ways. In males, the isolated germ cells could be autotransplanted to the testis or matured in vitro until they reach a stage sufficiently mature to achieve fertilisation with ICSI. Immature testicular tissue has been shown to grow and differentiate when grafted into another species.[35] This provides an additional strategy for conserving the male germ line and circumvents the risk of reintroducing malignant cells. Clearly this technique is unlikely to be ethically acceptable and is compromised by the risk of interspecies transfer of potentially pathogenic microorganisms. In females, one potential strategy for the future use of the stored material would be to replant the ovarian tissue, with the hope of restoring natural fertility and also maintaining sex steroid production.

Autologous transplantation of fresh and frozen thawed primordial follicles to the ovaries of sterile recipients has restored fertility, resulting in live healthy offspring in mice and sheep.[36] Human primordial follicles survive cryopreservation, and return of ovarian hormonal activity has been achieved with reimplantation.[37] However, no pregnancies have yet been reported and this procedure must be considered experimental. It is likely that ovarian grafts will have a limited life span, in which case transplantation should be delayed until fertility is desired. In females, harvesting ovarian tissue and cryopreservation of cortical slices are practiced in a number of centres, although these procedures remain experimental and future utilisation of stored tissue is uncertain. As with males, in view of the potential risk of transplanting tumour cells, a number of alternative strategies are being investigated. Isolation of follicles and in vitro maturation for assisted reproduction is likely to be the best option and may be the only option when the uterus has been damaged by radiotherapy.

There is a dearth of data reported on the experience of human ovarian tissue cryopreservation. In a study of 51 women, aged 17.9 (2.7–34) years, at risk of infertility secondary to treatment for cancer, ovarian tissue was harvested in 31 patients, 71% of whom had previously received chemotherapy.[38] In 77% of subjects the whole ovary was removed, while in the remainder half of the ovary was removed, either laparoscopically (n = 29) or at laparotomy (n = 2). Evaluation of the number of primordial follicles in the ovarian cortex showed a mean of 20/mm^2 in girls less than 7 years (n = 6), 4/mm^2 in girls aged 10–15 years (n = 8), diminishing to 1.6/mm^2 in girls greater than 15 years old. Of the 31 patients, 11 died, while follow up of the remainder was limited. Eight patients were lost to follow up and of the remaining 12 patients, no data were available on ovarian function. Although this study showed the feasibility of ovarian tissue cryopreservation, clearly structured prospective studies are required.[38]

ETHICAL ISSUES

Harvesting gonadal tissue and its future use is an exciting new area of gamete biology which raises a wide range of ethical and legal dilemmas that must be addressed before embarking on any clinical programme.[33 34 39–41] These include the safety of the tissue harvesting, subsequent use, and possible implications for the progeny, as well as the legal constraints enforced by the Human Fertilisation and Embryology Authority (HFEA), and the common laws defining validity of consent. For clinical research involving potentially harmful interventions, valid consent is necessary. To be valid, consent must be informed, voluntarily obtained, and given by a competent person. Legal competence to consent requires that the individual giving it is able to understand the information given, believes that it applies to them, retains it, and uses it to make an informed choice. The complex issues of fertility preservation and limited time for discussion imposed by therapeutic imperatives, further fuelled by parental and patient anxieties, will inevitably diminish the validity of such consent. Some of these practical difficulties may be alleviated if obtaining consent could be divided into two stages, with part one involving harvesting and cryopreservation of the tissue and part two involving subsequent use of the tissue. Issues relating to the use of the tissue in the event of the death of the patient must also be discussed.[34 39]

The legal framework defines who is eligible to give the consent. Adolescents over the age of 16 years in Scotland, and 18 years in England, may give consent to treatment in accordance with the Family Law Reform Act 1969 s8, while for younger children, "minors", consent is generally obtained by proxy. In exception to this, legally valid consent from "minors" can be obtained if their doctor considers that they are competent to make an informed decision (Gillick competence).[42] However, the field of assisted reproduction is governed by the statute in the UK and is under the jurisdiction of the HFEA (HFEA Act, 1990), which dictates strict guidelines on the requirements for informed consent with respect to the storage of gametes and embryos and their subsequent use.[43] The HFEA grants licences to individuals for certain procedures involving gametes. Proxy consent is specifically excluded and there is a requirement to provide written and verbal information and an offer of independent counselling. A gamete is defined by the HFEA to be "a reproductive cell, such as an ovum or spermatozoon, which has a haploid set of chromosomes and which is capable of taking part in fertilisation with another of the opposite sex to form a zygote".[44] In practice this would mean that non-gametes, or immature germ cells, could be harvested from children, with parental consent, and stored in, at present, unlicensed premises. If this immature material should ever be matured to form gametes, the requirements for storage of such material would then fall under the jurisdiction of the HFEA. In sexually mature boys, sperm may be produced or retrieved surgically, and written consent obtained in accordance with the Gillick principle and cryopreserved in a licensed centre.

Harvesting and storage of ovarian cortical tissue from girls and young women before gonadotoxic chemotherapy has been available in a number of centres since the mid-1990s and more recently, a few centres have reported the storage of testicular tissue.[38 45] The Royal College of Obstetricians and Gynaecologists has provided a report from a working party on the storage of ovarian and prepubertal testicular tissue. This provides standards for best practice in the cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures, and requirements for safe storage.[40]

In December 1999 an international conference was held in Cambridge, to develop an ethically acceptable strategy for the practice and research related to preserving fertility in children and adolescents being treated for cancer. From this meeting a consensus statement was drawn up which made a number of recommendations.[39] Integral to these recommendations were the design and implementation of well constructed research strategies, confined to a finite number of specialist centres, with centralisation of data and rapid dissemination of the results. In turn, these protocols and results should be subject to rigorous review to ensure high standards for collection procedures and storage of material. This would involve multidisciplinary teamwork with multicentre collaboration, to ensure that the best interests of the child are met. It was also recommended that prospective studies be set up to gather data on the impact of current treatment strategies on fertility outcomes in prepubertal children treated for cancer. The experimental nature of this work makes it essential to ensure that clinical and research practice develops in a phased and coordinated manner.

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Clinical Endocrinology (2003) **58**, 296–301

Is inhibin B a potential marker of gonadotoxicity in prepubertal children treated for cancer?

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(Received 12 July 2002; returned for revision 28 August 2002; finally revised 4 September 2002; accepted 27 September 2002)

Summary

BACKGROUND AND OBJECTIVE **Chemotherapy treatment of childhood cancer may impair gonadal function, which may be manifested only in adulthood as permanent sterility. Detection of gonadal dysfunction in prepubertal children has been hampered by the absence of a sensitive marker. Inhibin B is secreted by small antral follicles and Sertoli cells in females and males, respectively, and may be a marker of gonadal function in prepubertal children. The aim of this pilot study was to evaluate inhibin B in relation to sensitive measurements of gonadotrophins as markers of the early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer.**

STUDY DESIGN AND SUBJECTS **Twenty-five prepubertal children (9 females), median age 4·5 years (range 1·2–12·8 years) with cancer (16 solid tumours, nine acute lymphoblastic leukaemia, ALL) were studied longitudinally. Blood samples were collected before and during chemotherapy (solid tumours) or immediately following induction and first intensification (ALL). Post-treatment (1–6 months) samples were collected in 12 of the patients (5 females).**

MEASUREMENTS **Dimeric inhibin B was measured by double antibody enzyme-linked immunosorbent assay (ELISA). FSH and LH were measured by sensitive time-resolved immunofluorescence.**

RESULTS ***Girls*: Pretreatment inhibin B was slightly high in one girl but normal for age and sex in all others: median 16·1 (range 9·4–186·2) ng/l, median SD score +0·2 (–1·3 to +2·6). Inhibin B decreased to undetectable levels (< 8 ng/l) in 8/9 girls during treatment ($P = 0·03$), with no accompanying rise in FSH or LH. Post-treatment recovery of inhibin B was variable: median 16·1 (range < 8·0–44·2) ng/l, median SD score +0·1 (range < –2·4 to +1·8). Sustained undetectable inhibin B levels were observed in 2/5 girls with correspondingly elevated FSH concentrations (11·8 and 10·9 U/l).**

***Boys*: Inhibin B was normal for age and sex in all boys before treatment with no significant change during or after treatment (medians 93 ng/l, 85 ng/l and 94 ng/l, SD scores –0·3, –0·6 and –0·2, respectively). Inhibin B decreased to undetectable levels in one boy post-treatment with no accompanying increase in FSH or LH.**

CONCLUSIONS **In prepubertal girls with cancer, chemotherapy is associated with suppression of inhibin B, usually transient, which may indicate arrest of follicle development. Sustained suppression of inhibin B following treatment may be indicative of permanent ovarian damage. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed effect. Inhibin B, together with sensitive measurements of FSH, may be a potential marker of the gonadotoxic effects of chemotherapy in prepubertal children with cancer.**

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The successful treatment of childhood cancer may be associated with gonadal dysfunction and infertility in adulthood (Waring & Wallace, 2000; Thomson *et al.*, 2002). Chemotherapy and radiotherapy may damage testicular germinal epithelium resulting in temporary or permanent azoospermia in the male, or hasten oocyte depletion with truncated fecundity and a premature menopause in the female. Although chemotherapy may damage the gonads in both sexes throughout life, females appear to be less susceptible to the deleterious effects of chemotherapy than males. The extent of chemotherapy-induced damage is dependent upon the agent administered and dose received. However, it can be difficult to determine the relative contribution of each individual

drug as most treatments are administered as multiagent regimens. A number of agents have been identified as being gonadotoxic, including cisplatin, melphalan and the alkylating agents, such as cyclophosphamide.

Traditionally, determining the impact of chemotherapy and radiotherapy on gonadal function has involved clinical assessment of pubertal development, menstrual history in females and semen analysis in males. Earlier detection of gonadal damage has been hampered by the lack of a sensitive marker of gonadal function in prepubertal children. In these children gonadotrophins may be unreliable predictors of gonadal damage because the hypothalamic–pituitary–gonadal axis is relatively quiescent. Inhibin B is a dimeric glycoprotein produced by the gonads, secreted from Sertoli cells in males and developing antral follicles in females (Roberts *et al.*, 1993; Anderson & Sharpe, 2000). In the adult male inhibin B plays an important role in spermatogenesis, while in the adult female it plays an integral role in folliculogenesis, oocyte maturation and corpus luteal function, mediated in part by inhibition of FSH secretion. There is increasing evidence reporting that the prepubertal gonads are not entirely quiescent but have a low level of activity (Wu *et al.*, 1996; Kelnar *et al.*, 2002). We investigate the role of inhibin B, together with sensitive gonadotrophin measurements, as markers of early gonadotoxic effects of chemotherapy in prepubertal children treated for cancer.

Patients and methods

Patients

Stored plasma was analysed from blood samples collected for previous prospective, longitudinal studies exploring the effects of chemotherapy on growth and bone turnover (Crofton *et al.*, 1998; Bath *et al.*, in press). The original studies and their extension to this study were approved by the Lothian Paediatric and Reproductive Medicine Research Ethics Sub-Committee. The previous studies involved sequential recruitment of 47 children (18 females), median age 4·5 years (range 1·2–15·3 years), presenting with a variety of haematological and solid malignancies over a 2-year period (Crofton *et al.*, 1998; Bath *et al.*, in press). From this cohort we excluded postpubertal patients ($n = 7$), germ cell tumours ($n = 1$) and patients for whom there was insufficient plasma stored before or during treatment ($n = 14$). The study group consisted of 25 clinically prepubertal children (nine girls). The median age (range) at diagnosis was 4·5 (1·2–12·8) years and underlying malignancies included acute lymphoblastic leukaemia (ALL, $n = 9$, females 3), neuroblastoma ($n = 5$, females 1), non-Hodgkin's lymphoma ($n = 3$, females 0), rhabdomyosarcoma ($n = 3$, females 2), osteosarcoma ($n = 2$, females 1), Ewing's sarcoma ($n = 1$, females 1), medulloblastoma ($n = 1$, females 1) and Wilms ($n = 1$, females 0). Children with solid tumours ($n = 16$) were treated with multiagent chemotherapy regimens in accordance with United Kingdom Childrens Cancer Study Group (UKCCSG) protocols. One female also received lumbar radiotherapy for Ewing's sarcoma (L3). Children with ALL ($n = 9$) were treated with the MRC UKALL XI protocol. We collected blood samples from patients with solid malignancies ($n = 16$) before and during chemotherapy (median number of cycles 7, range 4–14) and from patients with ALL ($n = 9$) pretreatment and at the end of week 6 (after completion of induction and first intensification). In 12 of the 25 children treated for cancer, follow-up blood samples were collected between 1 and 6 months after completion of chemotherapy.

Methods

Blood samples were separated immediately after collection and plasma stored at −70°C until analysis. Dimeric inhibin B was measured in duplicate by a double antibody enzyme-linked immunosorbent assay (ELISA), using a monoclonal antibody raised against a synthetic peptide from the βB subunit, combined with an antibody to an inhibin α subunit sequence, as described (Groome *et al.*, 1996; Crofton *et al.*, 2002a). The assay detection limit was 8 ng/l. Within- and between-assay coefficients of variation (CVs) were 9·6% and 13·0% at 19 ng/l, 7·4% and 10·6% at 88 ng/l, and 8·4% and 10·2% at 233 ng/l, respectively. FSH and LH were measured in duplicate by Delfia time-resolved immunofluorescence assays (Wallac, Milton Keynes, UK), standardized against the second International Reference Preparations of pituitary FSH and LH, coded 78/549 and 80/552, respectively. Assay detection limits were 0·3 U/l for FSH and 0·2 U/l for LH. Between-assay CVs for FSH were 6·5% at 5·2 U/l, 3·5% at 10·4 U/l and 3·6% at 35·3 U/l, and for LH were 5·9% at 6·4 U/l, 5·5% at 11·3 U/l and 4·4% at 18·7 U/l.

Data analysis

Inhibin B varies with age and sex. Data are presented both as absolute concentrations (ng/l) and as SD scores calculated for age and sex following log-transformation, based on our own published data (Crofton *et al.*, 2002a,b). Group results are presented as medians and ranges. Within-individual changes through time were evaluated by Wilcoxon matched pairs.

Results

Girls

Inhibin B, FSH and LH results in the children before, during and after chemotherapy are shown in Table 1. Pretreatment inhibin B levels were comparable to the normal age- and sex-matched

Table 1 Median (range) of inhibin B, FSH and LH levels in children before, during and after chemotherapy

	Before	During	After
Girls			
*N**	9 (6)	9 (6)	5 (5)
Inhibin B (ng/l)	16·1 (9·4, 186·2)	< 8·0 (< 8·0, 20·8)†	16·1 (< 8·0, 44·2)
Inhibin B (SDS)	+0·2 (− 1·3, +2·6)	−‡	+ 0·1 (< −2·4, +1·8)
FSH (U/l)	0·7 (< 0·3, 1·9)	1·0 (< 0·3, 3·0)	6·0 (1·4, 11·8)
LH (U/l)	< 0·2 (< 0·2, 0·2)	< 0·2 (< 0·2, < 0·2)	< 0·2 (< 0·2, 1·1)
Boys			
*N**	16 (10)	16 (10)	7 (6)
Inhibin B (ng/l)	93 (77, 244)	85 (58, 261)	94 (< 8, 170)
Inhibin B (SDS)	−0·3 (− 1·9, +1·8)	−0·62 (−1·8, +1·8)	−0·18 (< −7·0, +1·4)
FSH (U/l)	< 0·3 (< 0·3, 1·2)	< 0·3 (< 0·3, 0·8)	0·4 (< 0·3, 2·6)
LH (U/l)	< 0·2 (< 0·2, 0·3)	< 0·2 (< 0·2, < 0·2)	< 0·2 (< 0·2, 0·3)

*Total number of children with cancer (number with solid tumours).
†$P < 0·05$ compared with pretreatment levels.
‡Not reported because 8/9 girls had undetectable inhibin B during chemotherapy (see text).
SDS, SD score according to age and sex.

population in eight of the nine girls. One clinically prepubertal girl, aged 10·5 years, had a high inhibin B level for age (SD score +2·6) but prepubertal gonadotrophin levels (FSH 1·9 U/l, LH 0·2 U/l). All other girls had pretreatment inhibin B levels within their respective age- and sex-specific reference ranges and appropriate for prepubertal girls (Crofton *et al.*, 2002a). FSH and LH concentrations were prepubertal in all nine girls.

During treatment, inhibin B decreased to undetectable levels in eight of the nine girls ($P = 0·03$ compared with pretreatment samples) (Fig. 1a) with no accompanying rise in FSH or LH (Table 1). The only girl in whom inhibin B was not suppressed was treated with low doses of potentially gonadotoxic agents (cyclophosphamide 1·5 g/m^2, cisplatin 80 mg/m^2 and carboplatin 1·0 g/m^2).

Samples were available from five girls after completion of treatment. Analysis of post-treatment samples demonstrated variable recovery of inhibin B, with levels remaining below the assay detection limit in two girls (Fig. 1a). FSH (but not LH) increased in all girls post-treatment compared with their pretreatment levels ($P = 0·06$, Table 1). The two girls (aged 2·6 and 10·5 years, respectively) in whom inhibin B remained undetectable post-treatment had the highest post-treatment levels of FSH (11·8 and 10·9 U/l, respectively).

Boys

Inhibin B levels were appropriate for age with no significant changes before, during and after treatment in all but one boy (Table 1). FSH and LH levels remained prepubertal in all patients pre-, during and post-treatment (Table 1). Following completion of treatment, six of the seven boys showed little change in inhibin B (Fig. 1b). In one patient (aged 2 years), inhibin B levels decreased to undetectable levels following completion of treatment with alkylating agent-based gonadotoxic chemotherapy (cyclophosphamide, 4·4 g/m^2, cisplatin 320 mg/m^2 and melphalan 200 mg/m^2) with no post-treatment increase in gonadotrophins (FSH 0·8 U/l, LH < 0·2 U/l).

Discussion

Girls

Chemotherapy treatment of childhood cancer is associated with suppression of inhibin B during treatment, which may indicate arrest of follicular development. Recovery of ovarian function following completion of chemotherapy treatment is variable and is likely to reflect the severity of the gonadotoxic insult. Some of the girls treated with known gonadotoxic agents demonstrated sustained suppression of inhibin B levels and continued assessment will be necessary to observe if this is transient or associated with impaired ovarian function and development of a premature menopause.

At diagnosis, prepubertal girls' inhibin B levels were appropriate for age and sex, indicating that they continued to maintain normal ovarian follicular development, at least to the small antral stage. During treatment with a variety of combination chemotherapy regimens, inhibin B was suppressed in all but one girl, indicating arrest of follicular development from the primordial pool. The exception was a girl in whom chemotherapy had been curtailed owing to lack of tumour response and who had therefore been exposed to relatively low doses of potentially gonadotoxic chemotherapy. There was no accompanying increase in FSH levels in the girls with suppressed inhibin B, consistent with hypothalamic-pituitary quiescence during chemotherapy in these prepubertal girls. Although all three girls with ALL had undetectable inhibin B at the end of first intensification (which

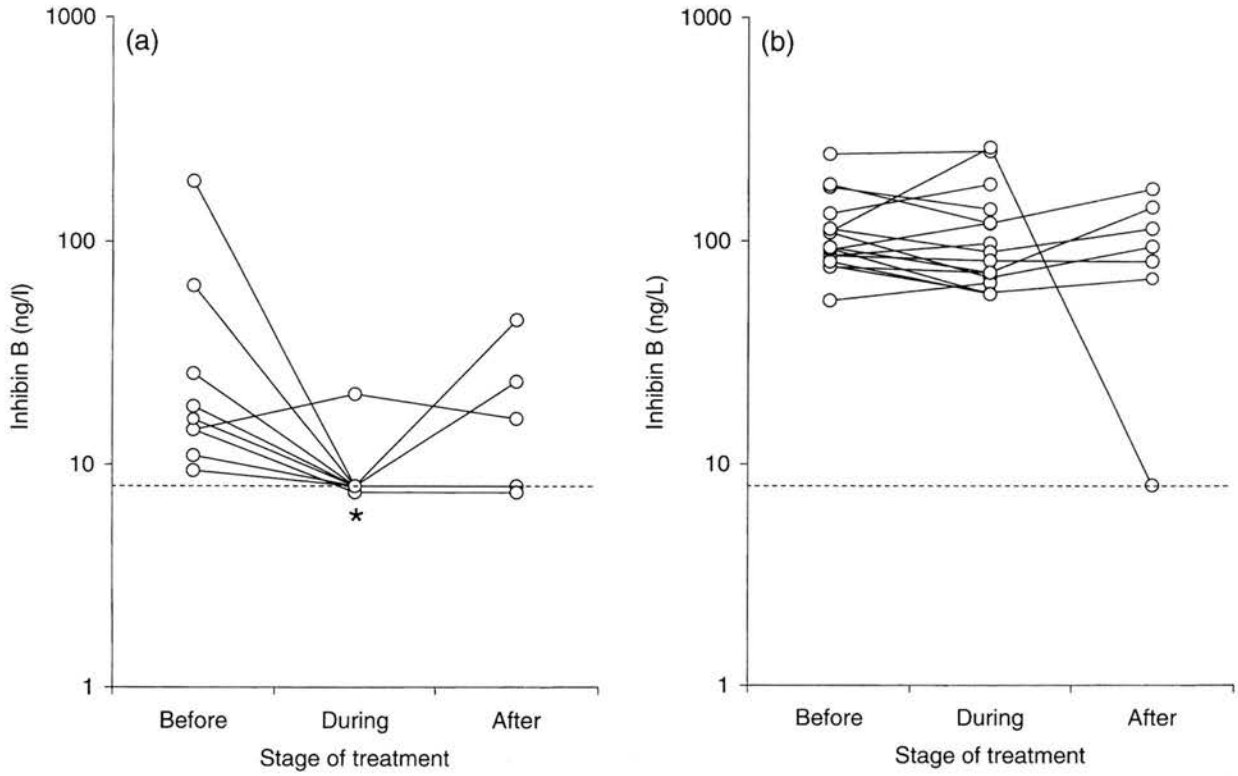

Fig. 1 Inhibin B levels in individual children with cancer before, during and after treatment: (a) girls; (b) boys. Inhibin B is plotted on a log scale. The dotted line represents the detection limit of the inhibin B assay. Results below the assay detection limit are plotted at the detection limit. $*P < 0{\cdot}05$ compared with pretreatment levels (Wilcoxon matched pairs). See text for details.

includes cytarabine, a potentially gonadotoxic agent), inhibin B returned to normal, detectable levels 2–4 weeks later and remained normal in all three girls during the second year of continuing chemotherapy (data not shown). These results suggest that arrest of folliculogenesis in girls treated for ALL is likely to be transient. This is consistent with retrospective studies of survivors of childhood ALL that have reported relatively normal ovarian function and reproductive pattern in females treated with chemotherapy (Nygaard *et al.*, 1991; Wallace *et al.*, 1993).

After completion of treatment, there was variable recovery of inhibin B. Two girls in whom inhibin B remained undetectable had elevated post-treatment levels of FSH, suggesting resumption of pituitary activity with loss of feedback suppression on FSH production. One of these girls was aged 10·5 years at the start of treatment and 11·3 years old at the time of follow-up, 6 months after completion of treatment, an age at which there is normally active follicular development to the small antral stage with detectable inhibin B in all girls (Crofton *et al.*, 2002a). This girl therefore appeared to have persistent defective folliculogenesis following gonadotoxic chemotherapy (cisplatin 600 mg/m^2). Cisplatin doses of this magnitude have been shown to be associated with long-term ovarian damage in survivors (Wallace *et al.*, 1989). Post-treatment inhibin B was also undetectable in a 2-year-old patient treated for neuroblastoma with combination chemotherapy including the gonadotoxic agents cisplatin (320 mg/m^2), cyclophosphamide (4·2 g/m^2), and melphalan (200 mg/m^2) as preconditioning therapy for autologous bone marrow transplantation. At this age inhibin B may be undetectable on random sampling in up to 35% of normal girls (Crofton *et al.*, 2002a). However, her FSH increased from 0·7 U/l pretreatment to 11·8 U/l post-treatment. The combination of undetectable inhibin B and increased FSH is suggestive of ovarian damage.

Although the numbers in our study are small, the data suggest that the arrest of follicular development associated with potentially gonadotoxic chemotherapy may persist in some girls during the early post-treatment period. In other girls, inhibin B and FSH levels were normal after completion of treatment, with a positive relationship between the two hormones, as observed in normal prepubertal girls (Crofton *et al.*, 2002a). This is consistent with resumption of follicular development under the influence of FSH. Long-term follow-up of all patients is warranted to monitor for the development of premature ovarian failure.

Boys

Inhibin B, produced from Sertoli cells, mediates nonsteroidal negative feedback from the testes, reflecting the number of spermatozoa produced and regulating FSH secretion (Pierik *et al.*, 1998). Inhibin B secretion in the adult requires the presence of germ cells and in the prepubertal boy may reflect continuous Sertoli cell proliferation and functional activity together with maturation of early germ cells and spontaneous degradation. Men with complete absence of germ cells (Sertoli cell only syndrome, SCOS), severe hypospermatogenesis and spermatogonial and/or spermacytic arrest have lower inhibin B levels and higher FSH levels than normozoospermic controls (Mahmoud *et al.*, 1998; Bohring & Krause, 1999; Foresta *et al.*, 1999). Gonadotoxic chemotherapy in men is associated with a decrease in circulating inhibin B during treatment (Wallace *et al.*, 1997). We have recently demonstrated reduced sperm concentration, reduced inhibin B and increased FSH levels in male survivors of childhood cancer (Thomson *et al.*, 2002). Chemotherapy has been implicated as the cause of these endocrine abnormalities in adult survivors of childhood cancer (Lähteenmäki *et al.*, 1999; Schmiegelow *et al.*, 2001). However, a longitudinal study of prepubertal boys with acquired SCOS caused by irradiation/chemotherapy for ALL has demonstrated that reductions in inhibin B levels are generally delayed until postpuberty (Andersson *et al.*, 1998).

In our study, boys with cancer had normal inhibin B levels before treatment started and these changed little during and after chemotherapy except for one boy with neuroblastoma treated with relatively high doses of known gonadotoxic agents, cyclophosphamide, cisplatin and melphalan (Watson *et al.*, 1985; Jaffe *et al.*, 1988; Aubier *et al.*, 1989; Wallace *et al.*, 1989; Chatterjee *et al.*, 1994; Waring & Wallace, 2000) in whom inhibin B became undetectable following completion of treatment, suggestive of Sertoli cell damage. This may reflect damage to the germinal epithelium and long-term follow-up and monitoring of testicular function will be essential. Our results show that inhibin B levels were normal in boys with ALL throughout treatment, including year 2 of continuing chemotherapy (data not shown), consistent with reports of normal testicular function and normal spermatogenesis in most male survivors of childhood ALL (Blatt *et al.*, 1981; Wallace *et al.*, 1991). FSH and LH remained low or undetectable in all prepubertal boys with cancer before, during and after treatment, illustrating their insensitivity as markers of gonadal damage before the onset of puberty. In the boy who developed undetectable inhibin B, there was no accompanying increase in FSH, consistent with previous evidence that inhibin B and FSH secretion are independent in prepubertal boys, in contrast to the inverse relationship that develops later during puberty (Crofton *et al.*, 2002b).

In summary, prepubertal girls treated with chemotherapy developed suppressed inhibin B levels indicating arrest of follicular development, with variable short-term recovery of folliculogenesis after completion of treatment. In prepubertal boys, chemotherapy had little immediate effect on Sertoli cell production of inhibin B, although one boy showed a delayed deleterious effect. This pilot study has not addressed the long-term implications for future fertility. Further studies are planned, combining inhibin B with FSH, LH and sex hormone measurements, to assess longer term reversibility and delayed effects, particularly as the children approach and progress through puberty.

Acknowledgements

We are grateful to the Jennifer Fund for their generous support of this study.

Dr S. F. Ahmed was involved in the collection of samples from the patients with ALL. We also thank Dr A. Thomas at the Royal Hospital for Sick Children, Edinburgh, for allowing her patients to be studied.

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Semen quality and spermatozoal DNA integrity in survivors of childhood cancer: a case-control study

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Summary

Background Treatment of childhood cancer can result in impaired spermatogenesis. Intracytoplasmic sperm injection (ICSI), however, can enable men to achieve fatherhood, and has focused attention on gamete integrity in men with oligozoospermia. Our aim was to assess testicular function in survivors of childhood cancer.

Methods We assessed testicular function in 33 survivors of childhood cancer and 66 age-matched controls. The median age at diagnosis and at the start of the trial was 10·0 years (range 2·2–16·9) and 21·9 years (16·5–35·2), respectively. We assessed pubertal staging, measured plasma sex steroid hormone concentrations, and analysed semen quality, including spermatozoal DNA integrity.

Findings Ten (30%) individuals were azoospermic and six (18%) oligozoospermic (sperm concentration $<20\times10^6$/mL). Sperm concentration was significantly lower in the non-azoospermic group than in controls (median $37{\cdot}1\times10^6$/mL, IQR $19{\cdot}7\times10^6$ to $89{\cdot}9\times10^6$, *vs* $90{\cdot}7\times10^6$/mL, $50{\cdot}5\times10^6$ to $121{\cdot}5\times10^6$; p=0·002). In the non-azoospermic cancer survivor group, inhibin B concentrations were lower than in controls (mean 153·3 ng/L, SEM 17·8, *vs* 223·7 ng/L, 8·8; p<0·001), and FSH concentrations were higher (6·6 U/L, 0·9, *vs* 3·2 U/L, 0·2; p<0·001). Only 11 (33%) survivors of childhood cancer had normal semen quality. There was no significant difference in sperm DNA integrity between the non-azoospermic and control groups (9%, 5–13, *vs* 11%, 7–16; p=0·06).

Interpretation Sperm concentration is reduced after treatment for cancer. However, the sperm produced seems to carry as much healthy DNA as those produced by the healthy population, suggesting that assisted conception can be considered as a treatment option for these men.

Lancet 2001; **360:** 361–367. Published online July 9, 2002
http://image.thelancet.com/extras/01art7042web.pdf

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Introduction

70% of individuals survive childhood cancer, and this proportion continues to increase. As such, attention is being focused on the lasting morbidity associated with radiation and chemotherapy treatment.[1] A frequent and psychologically traumatic late complication of cancer treatment is infertility. Cytotoxic chemotherapy drugs, especially alkylating agents, can produce long-lasting or permanent damage to the germinal epithelium, resulting in oligozoospermia or azoospermia.[2–8] The germinal epithelium is also sensitive to radiotherapy, and doses as low as 1·2 Gy can result in permanent sterility.[9] Recovery from surviving germ cells can happen but is unpredictable and often takes a long time.[7,8] Leydig cells, with their slower rate of turnover, are more resistant to gonadotoxic therapy, resulting in preservation of androgen production even when patients are infertile.[5]

Advances in techniques of assisted reproduction, especially intracytoplasmic sperm injection (ICSI), have enabled some men with oligozoospermia to become fathers.[10,11] Concerns have been raised, however, about the safety of ICSI,[12] since whether or not spermatozoa from men with impaired spermatogenesis carry abnormal genetic information is unkown.[13–15] Data on the health of offspring born after ICSI are broadly reassuring,[16] though there are no data on the health of children born to fathers whose deficit in semen quality is a specific consequence of potentially mutagenic treatment.[17] Results of studies in animals have shown that exposure of the male germ line to chemotherapy agents can disrupt spermatozoal DNA and result in deleterious effects on embryo development.[18] Awareness of the importance of the integrity of sperm DNA for accurate transmission of genetic material to the offspring has necessitated the development of new techniques to assess sperm characteristics in more detail.[19]

Our aim was to investigate testicular function and semen quality in survivors of childhood cancer.

Methods

Patients

We searched the oncology database at the Royal Hospital for Sick Children, Edinburgh, for all male survivors of childhood cancer aged older than 16 years, and identified 51 individuals between December, 1999, and June, 2001. We invited 45 of these men to participate in the study, and excluded six because they no longer lived in the area (five) or were on antidepressant medication (one). Six of the 45 men declined and six did not reply to the invitation. The 18 men who did not participate in our study were comparable for age, diagnoses, age at diagnosis, treatment regimens, and disease-free survival. 33 men participated in the study. For each study participant, we recruited two age-matched controls (n=66). The volunteers were recruited by means of advertisement in local media and through hospital out-patient clinics, and selected on the basis of the absence of any clinical evidence, on history or physical examination, of reproductive health problems. The Lothian Paediatric and Reproductive Medicine research ethics subcommittee approved the study, and all patients provided written informed consent.