Title:
How does Chemotherapy Treatment Damage the Prepubertal Testis?

Authors:
Caroline M Allen¹, Federica Lopes¹, Rod T Mitchell², Norah Spears¹

Addresses:
¹ Biomedical Sciences, University of Edinburgh, Edinburgh, EH8 9XD, United Kingdom.
² MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, EH16 4TJ, United Kingdom

*Corresponding Author: Norah Spears; E-mail: norah.spears@ed.ac.uk

Word Count: 8295 (excluding abstract & acknowledgements)

Abbreviations:
Abstract

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

The overall childhood cancer survival rate has increased substantially in recent decades, with the current 5-year survival rate at around 80%, compared to about 58% in the late 1970s (Miller et al., 2016). This marked advance, due in large part to improved chemotherapy treatments, has led to a growing population of long-term childhood cancer survivors. However, chemotherapy drugs do not exclusively target malignant cells, also eliciting side effects due to off-target damage to healthy tissues. Given this situation, research is increasingly focusing on preventing damage to healthy organs, to improve the quality of life for childhood cancer survivors. For younger patients, detrimental effects of treatment on fertility can be a major concern (Zebrack et al., 2004). This is particularly problematic for male survivors of childhood cancer, given the current lack of available fertility preservation treatments, since, unlike adult patients, these prepubertal patients do not yet produce mature spermatozoa that can be used for routine sperm cryopreservation (Wyns et al., 2010; Wallace, 2011; Mitchell et al., 2017).

Recently, centres have started to cryopreserve immature testicular tissue from prepubertal boys before the commencement of chemotherapy treatment. In 2015, there were seven centres in Europe collecting such tissues with more than 260 prepubertal samples stored, with biopsies only undertaken when treatment is deemed at high risk for later fertility complications (Picton et al., 2015; Mitchell et al., 2017). At this point, however, it is not yet certain if such cryopreserved tissue can be successfully used later to restore fertility in humans, as production
of viable sperm from such tissue has yet to be shown. A recent report has described the
generation of sperm-like cells after three-dimensional culture of isolated spermatogonial cells
obtained from testis biopsies taken from prepubertal boys undergoing chemotherapy
treatment (Abofoul-Azab et al., 2018). This is encouraging for the future, but in this preliminary
study, the technique was successful in only one out of six patient biopsies, and the functionality
of the sperm-like cells remains to be established. Research in animal models has been more
successful in showing the potential of fertility preservation techniques which could be
developed for clinical use (reviewed in Picton et al., 2015; Giudice et al., 2017). Transplantation
of spermatogonial stem cells (SSCs) or frozen-thawed immature testicular tissue grafted back
into the adult has been successful in producing functional gametes in animal models including
the non-human primate and murine (Brinster and Zimmermann, 1994; Mitchell et al., 2010;
Hermann et al., 2012; Jahnukainen et al., 2012). Healthy offspring have been produced through
IVF/ICSI using sperm derived from xenografted non-human primate immature testicular tissue
demonstrating the potential of this technique for clinical application (Liu et al., 2016). There are
concerns, however, for non-solid tumours that malignant cells could be reintroduced back into
the patient, particularly with tissue transplantation as shown in Hou et al., (2007) study where
leukemic infiltration was noted within the xenografted testicular tissue. In addition to in vivo
techniques, sperm has been grown in culture from immature testis through in vitro
spermatogenesis and these sperm have been used for IVF/ICSI to produce viable embryo’s in a
mouse model system (Sato et al., 2011). There have also been recent reports of in vitro culture
of human prepubertal testicular tissue, although without completion of spermatogenesis (de
Michele et al., 2017, 2018). For all such potential fertility preservation techniques, further
research is needed to allow the methods to be successfully transformed for use with human
tissue, after which further validation will be required to ensure that such methodologies are
efficient and safe for clinical use. An alternative strategy, rather than the subsequent
restoration of fertility after chemotherapy-induced infertility, would be the development of
interventions administered before and/or during chemotherapy treatment to prevent the
damage from occurring in the first instance, thus protecting the fertility potential of the patient.
Cytoprotective agents which specifically protect the prepubertal testis against chemotherapy-
induced damage, without interfering with the toxicity to cancer cells, could potentially be
employed as part of the chemotherapy treatment regimen. However, further research is
required to fully understand how chemotherapy drugs target the prepubertal testis and which
compounds could potentially prevent such damage.

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

2. Methodology

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

3. Childhood Cancer and Treatment

3.1 Incidence of Childhood Cancer

Each year around 1800 children are diagnosed with neoplastic disease, accounting for 1% of
new cancer diagnoses in the UK (Wallace, 2011; Cancer Research UK, 2015). Cancer is the
second most common cause of death in children. The nature of cancers that develop at this
young age differ from those of adult malignant tumours. In infants, tumours of embryonal
origin such as neuroblastomas are the most frequent cause, whereas in older children leukemia
(particularly acute lymphoblastic leukemia) as well as central nervous system tumours and
lymphomas (non-Hodgkins and Hodgkins) are more often diagnosed. There is also a sex
difference in incidence, with boys 1.2 times more likely to be diagnosed with cancer,
particularly lymphomas and central nervous system tumours (Kaatsch, 2010; Kelly, 2017).

3.2 Chemotherapy Treatment

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

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

4. Testis Development

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

4.1 Fetal Life

The testis forms during early fetal life from an undifferentiated bipotential gonad. The primordial germ cells, originally located outside the embryo within the yolk sac, migrate and populate the gonadal ridge (Stukenborg et al., 2014). Under the action of the SRY (sex determining region Y) gene, which drives production of the SOX9 (SRY-box 9) protein, Sertoli
cells differentiate from precursors cells within the gonadal ridge and engulf the primordial germ

cells which are now classified as gonocytes. This configuration results in the formation of

seminiferous cords which predominantly contain Sertoli cells with centrally located gonocytes.

The Sertoli cells are key drivers in the differentiation and function of other cellular components

of the testis including the Leydig cells and peritubular myoid cells (reviewed in Svingen and

Koopman, 2013) (Figure 2B). The hypothalamic-pituitary-gonadal axis (HPG) is active during

fetal life, producing the gonadotrophins follicle stimulating hormone (FSH) and luteinizing

hormone, which are essential for the formation, maturation and function of key somatic cells

including the Sertoli and Leydig cells, respectively (Stukenborg et al., 2018a)

During fetal development, the gonocytes actively proliferate into early neonatal life, and

differentiation of the gonocytes into pre-spermatogonia occurs asynchronously during fetal and

early postnatal life, with the gonocytes having greater proliferative activity than the pre-

spermatogonia. In the rodent model, however, the proliferation of the gonocytes ceases during

late fetal life and resumes during the early postnatal period (Ferrara et al., 2006; Mitchell et al.,

2008; Wu et al., 2009). Sertoli cells actively proliferate during fetal development under the

control of multiple hormones, particularly FSH. This period of proliferation is very important in

determining adult numbers of Sertoli cells (Sharpe et al., 2003). Peritubular myoid cells develop

from interstitial cells, when multiple layers of these cells along with extracellular matrix

proteins surround and form the wall of the seminiferous cords. These cells contribute to the

basement membrane providing structural support during testis development, and will function

in the adult to aid movement of mature sperm towards the lumen (Mayerhofer, 2013; Svingen
and Koopman, 2013). The endocrine somatic Leydig cells are situated within the interstitial compartment between seminiferous cords and are composed of different cell populations throughout an individual’s lifespan. The fetal Leydig cells (FLCs) produce testosterone throughout fetal life, which is essential for masculinization of the male reproductive system. These cells ultimately regress after birth and are replaced by adult Leydig cells (ALCs) during puberty (Habert et al., 2001; Chen et al., 2009; Svechnikov et al., 2010), however the presence and/or function of the FLCs have recently been shown to important in the formation and function of the ALCs in a rodent model (Su et al., 2018). Whether FLC and ALC are distinct population of cells or share a common stem/progenitor cell lineage is at present unknown, however stem ALCs have been observed in the human fetal testis (Kilcoyne et al., 2014; Teerds and Huhtaniemi, 2015).

4.2 Neonatal Life

During the first three months of neonatal life, the reproductive system undergoes a short period of activity known as “mini-puberty”, where the HPG axis is active (Kuiri-Hänninen et al., 2014). The origin of the Leydig cells that produce testosterone during ‘mini-puberty’ is unknown, possibly forming from precursor cells or from the FLCs (Svechnikov et al., 2010). The function of ‘mini-puberty’ has yet to be clarified, but is thought to be linked to later reproductive function in the adult (Copeland and Chernausek, 2016). This short neonatal activity has also been observed in the primate animal model during neonatal development but does not appear to occur in rodents, where infancy overlaps with early stages of pubertal development (Chemes, 2001; Kelnar, 2002).
The volume of the testis increases six fold during neonatal development due to proliferation of Sertoli cells stimulated by FSH secretion; 93-95% of the seminiferous tubule mass is attributed to the Sertoli cells at this stage (Müller and Skakkebaek, 1983; Rey et al., 1993; reviewed in Petersen and Söder, 2006). This increase in cell number results in an overall increase in seminiferous cord length but not diameter as each Sertoli cell maintains contact with the basement membrane, the cells spreading lengthwise (Chemes, 2001). Therefore, in a cross sectional area of the seminiferous cord it may appear as though the cell density remains stable or even decreases during this period of development which can be misleading. The resulting number of Sertoli cells will ultimately determine sperm production in the adult, with each Sertoli cell able to support a species-specific number of germ cells (Sharpe, 2001).

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

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

During prepuberty, the proliferative rate of the germ cell population is reduced in comparison with fetal development, nevertheless there is a three fold increase in the overall population during this time period (Müller and Skakkebaek, 1983). At this point, the SSC pool is represented by undifferentiated A spermatogonia composed of two populations of cells with a dark (A_{dark}) or pale (A_{pale}) appearance. A_{dark} spermatogonia are believed to represent the reserve stem cells with low mitotic activity, whilst the A_{pale} cells are actively proliferating. Upon differentiation, the A_{pale} spermatogonia differentiate to B spermatogonia which can be observed from four-to-five years of age (Paniagua and Nistal, 1984). Type B Spermatogonia will enter meiosis to form spermatozoa in the adult (Ehmcke et al., 2006; Stukenborg et al., 2014).

In rodents, A_{single} (A_s) spermatogonia actively proliferate to form two conjoined daughter cells known as A_{paired} (A_{pr}) cells which continuously divide forming A_{aligned} (A_{al}) chains (reviewed in de Rooij and Russell, 2000; Ehmcke et al., 2006). Occasionally, spermatogonia enter meiosis, leading to the infrequent observation of primary spermatocytes during prepuberty, however these cells quickly degenerate since the somatic cells are not mature enough to support full
spermatogenesis at this stage (Chemes, 2001). The diameter of the tubules is unaltered during prepubertal development, with the lumen expanding only later during puberty due to intense germ cell proliferation that results in expansion in tubule width (Chemes, 2001). Reference values based upon a systematic review and meta-analysis performed by Masliukaite et al., (2016) indicates that spermatogonia number per tubular cross section and density per area (cm$^3$) decrease during the first three years of life followed by a gradual increase up to six-to-seven years of age, plateauing up to age 11 when boys begin to enter puberty and numbers increase dramatically. The initial decrease may be a result of programmed cell death of the gonocytes that failed to migrate to the basement membrane earlier in development (Masliukaite et al., 2016).

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

5. Evidence of Chemotherapy-induced Direct Damage to the Prepubertal Testis

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

5.1 Human Studies

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

5.1.1 Assessment of Immediate Testicular Damage in Prepubertal Patients

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

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

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

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

5.2 Summary of Human Studies

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

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

5.3.1 Germ cell Effects

5.3.1.1 Alkylating & Alkylating-Like Agents

As in human studies, research to date using prepubertal testis of rodents points to alkylating and alkylating-like agents targeting the germ cell population in a dose- and time- dependent manner (Table 4). SSCs have been shown to be particularly sensitive to cyclophosphamide and cisplatin, with treatment inducing DNA damage in vitro which could ultimately activate cell
death pathways if not repaired by internal DNA repair systems (Marcon et al., 2010; Liu et al., 2014). DNA damage was noted following short term (24hrs) exposure to chemotherapeutics using an in vitro culture system of prepubertal (pnd 5) mouse testis (Smart et al., 2018).

Activation of the apoptotic pathway was shown by enhanced cleaved caspase (CC) 3 expression following cyclophosphamide and cisplatin treatment with the timings differing between the agents, followed by loss of germ cells hours later. Increased numbers of apoptotic spermatogonia and primary spermatocytes have also been observed in vivo immediately following cisplatin treatment during early puberty (pnd 30-45) in the rat and after a 45-day recovery period (Lirdi et al., 2008; Favareto et al., 2011). The length of the treatment rather than the cumulative doses appears to be involved in determining the degree of testicular damage (Velez de la Calle et al., 1989). Shorter treatment regimens reduce exposure of the testis to the chemotherapeutic agent, which could be more important than overall cumulative dose received.

5.3.1.2 Anthracyclines

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

5.3.1.3 Topoisomerase Inhibitors

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

5.3.1.4 Vinca Alkaloids & Antitumor

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

5.3.1.5 Combination Treatments

In a clinical setting, cancer is treated with a combination of chemotherapeutic agents. Different classes of drugs target cells through differing mechanisms which may have synergistic effects. The combined treatments reduce the chance of resistance and survival of cancerous cells, but have the possibility of leading to multiplicative off-target side effects on healthy cells. Relatively few animal studies have focused on combinations of drugs (Table 4). Using the GC-6spg cell line, Beud et al., (2017) showed that a combination of vincristine and doxorubicin enhanced levels of cell death in a dose-dependent manner in comparison to treatment with each drug individually. In contrast, exposure to a combination of bleomycin, cisplatin and etoposide had no additional impact on cluster size/area of mouse SSCs in culture (Marcon et al., 2010). Further investigations into common combinations are required to determine the relative gonadotoxicity, and whether the effects are synergistic and/or multiplicative.

5.3.2 Somatic cell Effects
Studies relating to impacts of chemotherapy treatment upon the somatic cells has mainly been limited to the Sertoli cell and includes studies which have focused on cyclophosphamide, cisplatin, doxorubicin and etoposide chemotherapy agents (Table 4). Treatment results in morphological damage including vacuolation of the cells and adluminal positioning (Velez de la Calle et al., 1988, 1989, Stumpp et al., 2006, 2008; Favareto et al., 2011; Brilhante et al., 2012). However, these cells do survive exposure as shown in Smart et al., (2018) where there was no overall change in cell numbers following cyclophosphamide, cisplatin or doxorubicin treatment in an in vitro model of prepubertal mouse testicular tissue. Damage to the Sertoli cells resulting in dysfunction could be the primary effect of such treatment and would have a significant impact on testis function. Indeed, decreased production of androgen binding protein following treatment with alkylating and alkylating-like agents has been reported in prepubertal rats (Velez de la Calle et al., 1988, 1989; Favareto et al., 2011). In vitro studies using primary cultures of rat Sertoli cells have also shown reduced transferrin production following cisplatin, doxorubicin and vincristine treatment (Nambu et al., 1995). Transferrin stimulates germ cell proliferation/differentiation by transferring iron to these cells (Sylvester and Griswold, 1994). A potential mechanism by which cyclophosphamide and doxorubicin induce damage specifically to the Sertoli cell has been proposed by in vitro studies of cultured immature Sertoli cells where enhanced levels of oxidative stress have been reported (Liu et al., 2012; Tremblay and Delbes, 2018) as well as damage to the cytoskeleton following cyclophosphamide treatment (Liu et al., 2012).
Impairment of Sertoli cell functionality, however, may be secondary and a result of primary injury to the germ cell population. An *in vivo* study looking at doxorubicin-induced damage has shown that the alterations in the morphology and function of these cells was more pronounced in early adulthood in comparison to time of treatment just before puberty (pnd 22). This suggests that the germ cells were the primary cells targeted by doxorubicin, with loss/damage to the germ cells ultimately impacting Sertoli cells as a secondary effect (Brilhante *et al.*, 2012).

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

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

5.4 Summary of Animal Studies

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

6. Effects on Future Generations
The clinical impact of prepubertal chemotherapy treatment on later fertility has been discussed in several reviews (Hudson, 2010; Lee and Shin, 2013); for survivors who are able to conceive there could be potential effects upon future generations due to unrepaird damage to the male germline. The impact on future generations is not yet clear as a Danish study of 472 survivors of childhood cancer found no significant association between alkylating chemotherapy treatment and later genetic diseases of the progeny of these survivors (Winther et al., 2012).

Nonetheless, a study by Liu et al., (2014) has shown a potential mechanism by which alkylating agents can impact on future generations by targeting the telomeres of mouse spermatogonial cells. Telomerase function was reduced at concentrations of a drug precursor of cyclophosphamide (4OOH-CPA) and cisplatin which induced significant spermatogonial cell death resulting in reduced telomere length and activity of telomerase. A reduction in the length or function of telomeres of the male germ cell can adversely affect early development of the offspring, increasing the rate of pre- and/or post-implantation loss, congenital malformation and miscarriage (Liu et al., 2002). Whether additional classes of drugs also impact on future generations is unknown and therefore further research into this area is urgently required.

7. Protective Strategies

The development of chemotherapeutic treatment regimens has increased greatly in the past decade resulting in a greater number of childhood cancer survivors reaching adulthood and facing long-term consequences of treatment such as infertility. There is therefore an increasing focus on preserving the fertility of children undergoing cancer treatment. At the time of writing,
though, fertility preservation strategies for prepubertal boys have yet to be established clinically. Testicular tissue from boys undergoing ‘high’ risk chemotherapy treatment is being collected in a limited number of centres, with tissue cryopreserved and stored for potential fertility restoration when the boys reach adulthood. The proposed techniques to restore fertility, however, are still in the experimental phase of development with success to date only in rodent models (Picton et al., 2015; Giudice et al., 2017). Studies with human tissues have been conducted in relation to transplantation of cryopreserved testicular tissue and SSCs as well as in vitro maturation of immature testicular tissue paving the way for the development of a fertility restoration method clinically (reviewed Giudice et al., 2017). Xenotransplantation of prepubertal human testicular tissue for up to 9 months has been performed with nude mice where initiation of spermatogenesis was observed within the grafted tissue, however spermatogenesis did not reach meiotic differentiation (Wyns et al., 2007, 2008; Goossens et al., 2008; Van Saen et al., 2011, 2013). SSCs isolated from prepubertal patients have been propagated in vitro, but results are preliminary (Sadri-Arekani et al., 2011). Human prepubertal testicular tissue has been successfully cultured in vitro with survival of the spermatogonial germ cell population, maturation of the somatic cells and formation of the blood-testis barrier reported (de Michele et al., 2017, 2018). Initiation of spermatogenesis from such tissue has yet to be demonstrated. Alternatively, the prepubertal testis could be protected against injury caused by the treatment through use of cytoprotective agents that would be added to the cancer treatment regimen. Several studies have shown the gonadoprotective potential of agents in animal studies focusing on adult males, mainly investigating the morphology and motility of sperm. For example, Carmely et al., (2009) has shown in a mouse model the
cytoprotective effect of the immunomodulator AS101 compound against cyclophosphamide induced testicular damage. In contrast, so far only a limited number of compounds have been analysed to determine their ability to protect the prepubertal testis in an animal model (Table 5).

7.1 Amifostine

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

7.2 Carnitine

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

7.3 Ginseng Intestinal Metabolite I (GIM-I)

The herbal root, Ginseng has been used in East Asian countries as a traditional Chinese medicine and the intestinal metabolite, known as ginseng intestinal metabolite I (GIM-I), is thought to have multiple pharmacological effects through its antioxidant activity (Zhang et al., 1996). The protective effect against doxorubicin-induced damage has been analysed by Kang et al., (2002) in prepubertal mice in vivo, where GIM-I was found to partially protect against doxorubicin-induced germ cell damage resulting in testicular morphology comparable to controls that may be the result of enhanced antioxidant activity. GIM-I increases the levels of testis-specific antioxidants which were reduced following doxorubicin treatment. This compound has great potential as a cytoprotective agent against doxorubicin induced-damage and appears to have anti-metastatic activity and could therefore be added to chemotherapeutic regimens to provide benefit as both a cytotoxic and cytoprotective agent (Hasegawa et al., 1997).

7.4 Vitamin C and Curcumin

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

7.5 Summary of Protective Strategies

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

8. Conclusion

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

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

**Acknowledgements**

Funding by; Children with Cancer UK (grant #15–198). RM's work was undertaken in the MRC Centre for Reproductive Health funded by MRC Centre Grant MR/N022556/1, and CA was supported by Career Development PhD Scholarship in Biomedical Sciences funded by the Biomedical Sciences ZJU project. Thanks to Kathleen Duffin for comments on an earlier draft.

**Declaration of interest**
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Norah Spears is a member of the Editorial Board of Reproduction.

References


Brilhante O, Okada FK, Sasso-Cerri E, Stumpp T and Miraglia SM (2012) Late morfofunctional
alterations of the Sertoli cell caused by doxorubicin administered to prepubertal rats. 

Reproductive Biology and Endocrinology : RB&E 10 79.


Frederick NN, Recklitis CJ, Blackmon JE and Bober S (2016) Sexual Dysfunction in Young Adult


Copyright © 2018 Society of Reproduction and Fertility
cryopreserved testicular tissue preserves the fertility of prepubescent monkeys that receive sterilizing cytotoxic therapy. *Cancer Research* **72** 5174–5178.


Kuiri-Hänninen T, Sankilampi U and Dunkel L (2014) Activation of the hypothalamic-pituitary-


Liu M, Hales BF and Robaire B (2014) Effects of Four Chemotherapeutic Agents, Bleomycin, Etoposide, Cisplatin, and Cyclophosphamide, on DNA Damage and Telomeres in a Mouse


Rebours D, Shaughnessy PJO, Pitetti J, Monteiro A, Hara LO, Milne L, Tsai YT, Cruickshanks


leukaemia and spermatogonial stem cells. *Andrologia* 48 584–594.


Table and Figure legends

Table 1: Overview of Commonly used Chemotherapeutic Agents in Childhood Cancer Treatment Regimen.

Table 2: Human studies reporting Cyclophosphamide-induced Gonadotoxicity: Assessment of Immediate Testicular Damage.

Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient’s sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.

Table 3 Human studies reporting Cyclophosphamide-induced Gonadotoxicity: subsequent Assessment in the Adult.

Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient’s sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.

Table 4: Chemotherapy-induced Gonadal Toxicity - Animal Studies.
Gonadotoxicity determined with different classes either in isolation or combination. Results suggest that chemotherapy-induced damage is dependent on the chemotherapeutic agent, cumulative dose, stage of development and treatment regimen.

Table 5: Potential Cytoprotective Agents to Protect the Prepubertal Testis against Chemotherapy-induced Damage.

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

Figure 2: Comparison of Testicular Development in Humans and Rodents.
A) Relative timeframe of important developmental processes taking place between fetal development and puberty in humans (Chemes, 2001) and the mouse model (Vergouwen et al., 1993). Solid line indicates no activity of the cells at the relative time points and dashed line represent the unknown nature of Leydig cell development during this timeframe B) Comparison of the histology of the testis throughout development in the human, from fetal development through to the adult testis. ALC; adult Leydig cells, dpc; days post coitum, FLC; fetal Leydig cells, GC; germ cells GW; gestational week, LC; Leydig cells, PMC; peritubular myoid cells, pnd; postnatal days, SC; Sertoli cells.
<table>
<thead>
<tr>
<th>Chemotherapy Drug Class</th>
<th>Childhood Cancer Usage</th>
<th>Mechanism of Action</th>
<th>Cell Cycle Phase</th>
<th>Compounds</th>
<th>Current prediction of Infertility Risk *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating and Alkylating-Like Agents</td>
<td>Bone Cancer CNS Tumors Hodgkin's Lymphoma Kidney Cancer Leukemia Lymphoma Neuroblastoma Non-Hodgkin's Soft Tissue Sarcoma</td>
<td>Alkyl groups intercalate into nucleic acids and proteins. Intercalate into DNA by binding to the guanine or cytosine bases, resulting in crosslinks which disrupt DNA replication/transcription.</td>
<td>Non-specific</td>
<td>Carboplatin Chlorambucil Cisplatin Cyclophosphamide Ifosfamide Mechlorethamine Melphalan Oxaliplatin Procarbazine</td>
<td>Moderate High (&gt;1.4g/m²) High (&gt;0.6g/m²) High (&gt;7.5g/m²) High (&gt;60g/m²) High High (0.14g/m²) Moderate High (&gt;4g/m²)</td>
</tr>
<tr>
<td>Anthracyclines</td>
<td>Bone Cancer Hodgkin's Lymphoma Kidney Cancer Leukemia Lymphoma Neuroblastoma Non-Hodgkin's Soft Tissue Sarcoma</td>
<td>Target topoisomerase II, intercalate into DNA and producing free radicals</td>
<td>M-phase</td>
<td>Daunorubicin Doxorubicin</td>
<td>Unknown Moderate</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Bone Cancer Leukemia Lymphoma Non-Hodgkin's</td>
<td>Disrupt DNA/RNA synthesis</td>
<td>S-phase</td>
<td>Cytarabine Fluorouracil Mercaptopurine Methotrexate Thioguanine</td>
<td>Moderate Unknown Low Low Unknown</td>
</tr>
<tr>
<td>Taxanes</td>
<td>Ewing Sarcoma</td>
<td>Inhibit disassembly of microtubules</td>
<td>G₂/M-interphase</td>
<td>Docetaxel Paclitaxel</td>
<td>Unknown Unknown</td>
</tr>
<tr>
<td><strong>Topoisomerase Inhibitors</strong></td>
<td><strong>Bone Cancer</strong></td>
<td><strong>Target either topoisomerase I or II to disrupt DNA replication</strong></td>
<td><strong>M or S-phase</strong></td>
<td><strong>Etoposide</strong></td>
<td><strong>Unknown</strong></td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------------</td>
<td>---------------------------------------------------------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Vinca Alkaloids</strong></td>
<td><strong>Bone Cancer</strong></td>
<td><strong>Inhibit assembly of microtubules</strong></td>
<td><strong>M-phase</strong></td>
<td><strong>Vinblastine</strong></td>
<td><strong>Low</strong></td>
</tr>
<tr>
<td></td>
<td><strong>CNS Tumor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Hodgkins Lymphoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Kidney Cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Leukemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Lymphoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Neuroblastoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Non-Hodgkins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Soft Tissue Sarcoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Currently considered risk of infertility adapted from Wyns et al., (2010).
Table 2 Human studies reporting Cyclophosphamide-induced Gonadotoxicity: Assessment of Immediate Testicular Damage. Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient’s sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.

<table>
<thead>
<tr>
<th>Cyclophosphamide Dosage</th>
<th>Other drugs co-administered</th>
<th>Treatment Length</th>
<th>Male patients</th>
<th>Effect of Cyclophosphamide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>Age, years</td>
<td>Testicular Histology</td>
</tr>
<tr>
<td>Grams/Kg body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.002g/kg/day</td>
<td>Unknown</td>
<td>3 mo</td>
<td>1 (prepubertal: 1; CYC treated: 1)</td>
<td>6</td>
<td>Abnormal, atrophic tubules</td>
</tr>
<tr>
<td>0.003-0.024g/kg/day</td>
<td>Unknown</td>
<td>&lt;50-400 days</td>
<td>7 (prepubertal: 7; CYC treated: 7)</td>
<td>3-11</td>
<td>Normal</td>
</tr>
<tr>
<td>0.475-0.846g/kg</td>
<td>Asparaginse Cytarabine</td>
<td>2-6 y</td>
<td>46</td>
<td>0.08-13</td>
<td>Abnormal</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin Mercaptopurine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methotrexate Prednisolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vincristine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Copyright © 2018 Society of Reproduction and Fertility
<table>
<thead>
<tr>
<th>Dose range</th>
<th>Drugs</th>
<th>Age</th>
<th>Abnormal GCs</th>
<th>Reduced TFI</th>
<th>Complete loss of GCs CYC treated</th>
<th>Present</th>
<th>N/A</th>
<th>N/A</th>
<th>Study source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12-8.5 g/m²</td>
<td>Asparaginase, Bleomycin, Carmustine, Cytarabine, Dacl bazine, Dactinomycin, Daunorubicin, Dactinomycin, Doxorubicin, Fluorouracil, Lomustine, Mercaptopurin e, Methotrexate, Mitomycin C, Nitrogen Mustard, Prednisone, Procarbazine, Teniposide, Vinblastine, Vincristine, Vindesine</td>
<td>0.08 – 1 y</td>
<td>&lt;11</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Matus-Ridley et al., 1985</td>
<td></td>
</tr>
<tr>
<td>0.5 – 1.2 g/m²</td>
<td>Asparaginase, Cytarabine, Daunorubicin, Doxorubicin, Mercaptopurin e, Methotrexate, Prednisone, Vincristine</td>
<td>1.2–88.8 mo</td>
<td>9.4-16.6</td>
<td>Complete loss of GCs CYC treated</td>
<td>N/A</td>
<td>N/A</td>
<td>Müller et al., 1985</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1 g/m²</td>
<td>Asparaginase, Cytarabine, Doxorubicin</td>
<td>12-77 mths</td>
<td>3.5-15</td>
<td>Reduced TFI (&lt;40%) &gt;1 g/m² CYC</td>
<td>Present</td>
<td>N/A</td>
<td>Lendon et al., 1979</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Ages</td>
<td>Pubertal Status</td>
<td>FSH</td>
<td>Recovery</td>
<td>Notes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>-----------------</td>
<td>-----</td>
<td>----------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercaptopurine Methotrexate Prednisolone Vincristine</td>
<td>~1.8 yrs</td>
<td>37 (prepubertal: 37; CYC treated: 16)</td>
<td>1.1-16.1</td>
<td>N/A</td>
<td>Depletion of spermatogonial pool with reduced TFI (19%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytarabine Dexamethasone Doxorubicin L-Asparaginase Mercaptopurine Methotrexate Prednisolone Thioguanine Vincristine</td>
<td>3-4 yrs</td>
<td>25 (prepubertal: 24)</td>
<td>1.23-12.35</td>
<td>Abnormal</td>
<td>Complete loss or depletion of GC pool. Normal SC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparaginase Cytarabine Daunorubicin Hydroxyurea Lomustine Methotrexate Prednisolone Thioguanine Vincristine</td>
<td>6.6 - 7.6 yrs</td>
<td>23 (prepubertal: 23; CYC treated: 6)</td>
<td>2.8-8.6</td>
<td>N/A</td>
<td>Depletion in SSCs (↓ CD9 &amp; OCT4) and more differentiated spermatogonia (↓ MAGE4). Recovery noted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nurmio et al., 2009a
<table>
<thead>
<tr>
<th>Total Dose</th>
<th>Asparaginase</th>
<th>Cytarabine</th>
<th>Daunorubicin</th>
<th>Dexamethasone</th>
<th>Enocitabine</th>
<th>Hydrocortisone</th>
<th>Mercaptopurine</th>
<th>Methotrexate</th>
<th>Prednisolone</th>
<th>Vincristine</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-16.8g/m²</td>
<td>Unknown</td>
<td>12 (CYC treated: 7)</td>
<td>1-12</td>
<td>N/A</td>
<td>Reduced TFI (&lt;50%) with morphological changes to GCs and SCs but not linked to CYC</td>
<td>Present</td>
<td>N/A</td>
<td>Kobaya shi et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4-20.8g</td>
<td>Asparaginase</td>
<td>Cytarabine</td>
<td>Doxorubicin</td>
<td>Mercaptopurine</td>
<td>Methotrexate</td>
<td>Prednisolone</td>
<td>Vincristine</td>
<td>~4.4 yrs</td>
<td>37 (CYC treated: 14)</td>
<td>1.6-14.3</td>
</tr>
<tr>
<td>1.4-11.8g</td>
<td>Adriamycin</td>
<td>Asparaginase</td>
<td>Cytarabine</td>
<td>Mercaptopurine</td>
<td>Methotrexate</td>
<td>Prednisolone</td>
<td>Vincristine</td>
<td>30-81 mths</td>
<td>35 (prepubertal: 29; CYC treated: 5)</td>
<td>4.9-13.7</td>
</tr>
<tr>
<td>7.79g</td>
<td>Prednisolone</td>
<td>180 days</td>
<td>1 (prepubertal: 1; CYC treated: 1)</td>
<td>3-4</td>
<td>Normal</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal</td>
<td>Berry et al., 1972</td>
<td></td>
</tr>
</tbody>
</table>

CYC; cyclophosphamide, GC: germ cells, LC; Leydig cells, m0; months, y; years, TFI; tubular fertility index, SCO; Sertoli cell only tubules, SC; Sertoli cells, SSC; spermatogonial stem cells. # Information not included in study
**Table 3** Human studies reporting Cyclophosphamide-induced Gonadotoxicity: subsequent Assessment in the Adult. Studies suggest that the cumulative cyclophosphamide dose, age at treatment and patient’s sensitivity as well as the treatment regimen itself can influence the level of damage. As these patients often received a combination of chemotherapy drugs it is hard to determine the relative contributions of each drug. Studies were included only where the cyclophosphamide dosage and age of patient at time of treatment were known.

<table>
<thead>
<tr>
<th>Cyclophosphamide Dosage</th>
<th>Other drugs co-administered</th>
<th>Treatment Length</th>
<th>Patients within the Study</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams per body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2-0.5g/kg/day</td>
<td>Unknown</td>
<td>1.5 – 6 mo</td>
<td>23 (prepubertal: 16; CYC treated: 23)</td>
<td>Abnormal histology 20-25% of tubules atrophic high cumulative doses</td>
<td>N/A</td>
</tr>
<tr>
<td>0.104- 0.2g/kg</td>
<td>Prednisone</td>
<td>49-60 days</td>
<td>4 (CYC treated:4)</td>
<td>N/A</td>
<td>Normal testis morphology</td>
</tr>
<tr>
<td>0.312 – 1.325g/kg</td>
<td>N/A</td>
<td>89-489 days</td>
<td>4 (CYC treated:4)</td>
<td>N/A</td>
<td>Damage to seminiferous epithelium with SCOs</td>
</tr>
<tr>
<td>0.475-0.846g/kg</td>
<td>Doxorubicin Cytarabine L-Asparaginase Mercaptopurine Methotrexate Prexisolone Vincristine</td>
<td>2 to 6 y</td>
<td>46</td>
<td>Tubular damage occasionally observed 4 y after treatment with unknown “low” doses</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Grams/area
<table>
<thead>
<tr>
<th>Total Dose</th>
<th>Drugs</th>
<th>Total Dose</th>
<th>Morphology</th>
<th>Abnormal Morphology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6-29g/m²</td>
<td>Cytarabine, Dactinomycin, Daunomycin, Doxorubicin, Fluorouracil, L-Asparaginase, Mechloroethamine, Mercaptopurine, Procarbazine, Vincristine</td>
<td>30 (prepubertal: 19; CYC treated: 10)</td>
<td>1.75-17</td>
<td>N/A</td>
<td>Abnormal morphology</td>
</tr>
<tr>
<td>2.75-7.5g/m²</td>
<td>Asparaginase, Carmustine, Cutosine, arabinoside, Doxorubicin, Mercaptopurine, Methotrexate, Prednisone, Thioguanine, Vincristine</td>
<td>17 (prepubertal: 17; CYC treated: 5)</td>
<td>2.5-12.4</td>
<td>Reduced number of GC mm³</td>
<td>N/A</td>
</tr>
<tr>
<td>0.021 to 39g</td>
<td>Fluoxymesterone, Mercaptopurine Oxandrolone, Prednisone</td>
<td>9 wks -19 mo</td>
<td>7 (CYC treated: 7)</td>
<td>11-16</td>
<td>High doses; SCOs, peritubular fibrosis, normal morphology LCs. Low doses; active spermatogenesis 90% of tubules.</td>
</tr>
</tbody>
</table>

CYC; cyclophosphamide, GC: germ cells, LC; Leydig cells, wks, weels; mo; months, y; years, TFI; tubular fertility index, SCO; Sertoli cell only tubules, SC; Sertoli cells, SSC; spermatogonial stem cells. # Information not included in study
### Table 4

<table>
<thead>
<tr>
<th>Chemotherapy Drug</th>
<th>Doses (Length of Treatment)</th>
<th>Animal Model &amp; Age</th>
<th>In vivo or In vitro</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Alkylating and Alkylating-Like Agents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cisplatin</strong></td>
<td>10-15µg/ml</td>
<td>Mouse SSCs (isolated from pnd3-6)</td>
<td>In vitro</td>
<td>↓survival of SSC clusters in dose-dependent manner.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.1, 0.5 and 1µg/ml (24 hrs)</td>
<td>Prepubertal mouse (pnd5)</td>
<td>In vitro</td>
<td>↓GCs, particularly SSCs ↑CC3 expression 24 hrs after treatment, ↓GCs eight hrs later. ↑γH2AX GC expression 16 hrs after treatment. No significant effect on SC or LC numbers.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1µM (48 hrs)</td>
<td>Rat SSCs (isolated from pnd7-8)</td>
<td>In vitro</td>
<td>Dose-dependent ↑γH2AX.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.5µg/ml (24 hrs)</td>
<td>SCs prepubertal rat (pnd18)</td>
<td>In vitro</td>
<td>N/A</td>
<td>50%↓ transferrin production.</td>
</tr>
<tr>
<td></td>
<td>200 or 400µM (24 hrs)</td>
<td>SCs prepubertal rat (pnd19)</td>
<td>In vitro</td>
<td>N/A</td>
<td>↓cell viability (200µM) but no ↑CC3 activation at 24 hrs.</td>
</tr>
<tr>
<td></td>
<td>0.005g/kg</td>
<td>Pubertal rat (pnd30)</td>
<td>In vivo</td>
<td>↓seminiferous epithelium. ↑apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes 12 hrs after treatment.</td>
<td>N/A</td>
</tr>
<tr>
<td>Treatment</td>
<td>Dose</td>
<td>Species</td>
<td>Route</td>
<td>Findings</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>---------</td>
<td>-------</td>
<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td>0.001g/kg/day (3 wks)</td>
<td>Pubertal rat (pnd45)</td>
<td>In vivo</td>
<td>↑apoptotic, TUNEL positive pre-meiotic GCs and primary spermatocytes.</td>
<td>Vacuolation of SC. ↓ABP production.</td>
<td>Favareto et al., 2011</td>
</tr>
<tr>
<td>0.1µM (48 hrs)</td>
<td>C18-4 spermatogonial cells (mouse)</td>
<td>In vitro</td>
<td>↑γH2AX telomeres. ↓telomere length and activity of telomerase.</td>
<td>N/A</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.04-0.28g/kg (2 wks) 0.7g/kg total</td>
<td>Prepubertal rat (pnd10)</td>
<td>In vivo</td>
<td>No evidence of testicular damage.</td>
<td>N/A</td>
</tr>
<tr>
<td>Cyclophosphamide (4-hydroperoxy cyclophosphamide -4OOH-CPA metabolite)</td>
<td>0.002g/kg/day (5 wks) 0.7g/kg total</td>
<td>Pubertal rat (pnd45)</td>
<td>In vivo</td>
<td>Atrophied seminiferous tubules ↓spermatogonia and primary spermatocytes.</td>
<td>Vacuolation of SC. ↓ABP production.</td>
</tr>
<tr>
<td>Cyclophosphamide (arcolein metabolite)</td>
<td>0.1µM (48 hrs)</td>
<td>C18-4 spermatogonial cells (mouse)</td>
<td>In vitro</td>
<td>↑γH2AX telomeres expression. ↓telomere length and activity reduced.</td>
<td>N/A</td>
</tr>
<tr>
<td>Cyclophosphamide (phosphoramidate mustard metabolite)</td>
<td>50 or 100µM (3 or 12 hrs)</td>
<td>Mouse SCs (isolated from pnd8)</td>
<td>In vitro</td>
<td>↓viability of SC. ↑ROS and ↓antioxidant activity ↑apoptosis. Impairs cytoskeleton of SCs.</td>
<td>N/A</td>
</tr>
<tr>
<td>Cyclophosphamide (arcolein metabolite)</td>
<td>0.02, 0.2 and 2µg/ml (24 hrs)</td>
<td>Prepubertal mouse (pnd5)</td>
<td>In vitro</td>
<td>↓GCs, particularly SSCs ↑CC3 expression 16 hrs after treatment, ↓GCs eight hrs later. ↑γH2AX GC expression 16 hrs after treatment.</td>
<td>No effect on SC or LC numbers.</td>
</tr>
<tr>
<td>Procarbazine</td>
<td>0.030g/kg/day (5 or 9 wks)</td>
<td>Prepubertal (pnd10), pubertal (pnd45) &amp; adult rat (pnd70-90)</td>
<td>In vivo</td>
<td>5 wks; ↓diameter of SCO (prepubertal), less of an effect on pubertal and adults 9 wks; less impact on pubertal rats.</td>
<td>Vacuolation of SC. ↓ ABP production.</td>
</tr>
<tr>
<td>B) Anthracyclines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>40 and 100ng/ml (up to 72 hrs)</td>
<td>Prepubertal rat (pnd5)</td>
<td>In vitro</td>
<td>↓GC numbers 24 and 48 hrs, ↓proliferating and ↑apoptotic GCs, after 16 hrs.</td>
<td>No effect SCs number, production of key proteins. No impact on PMC proliferation or LC testosterone production.</td>
</tr>
<tr>
<td></td>
<td>0.05, 0.1 and 0.5µg/ml (24 hrs)</td>
<td>Prepubertal mouse (pnd5)</td>
<td>In vitro</td>
<td>↓GCs, particularly SSCs. No expression of CC3 observed before GC loss. ↑γH2AX GC expression 16 hrs after treatment.</td>
<td>No effect on SC or LC numbers.</td>
</tr>
<tr>
<td></td>
<td>0.003g/kg</td>
<td>Prepubertal (pnd6 &amp; 16) &amp; pubertal rat (pnd24 &amp; 45)</td>
<td>In vivo</td>
<td>Prepubertal rats ↑SSC death. Toxicity reduced in pubertal rats treated 3, 7 and 14 days after treatment.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.003g/kg</td>
<td>Prepubertal rat (pnd6, 16 and 24)</td>
<td>In vivo</td>
<td>Targets migrating gonocytes. ↑apoptotic cells in pnd6 rats 48 hrs after treatment, ↑p53 &amp; CC8 expression.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.4µg/ml (24 hrs)</td>
<td>SCs prepubertal rat (pnd18)</td>
<td>In vitro</td>
<td>N/A</td>
<td>35% ↓transferrin production.</td>
</tr>
<tr>
<td></td>
<td>0.005g/kg</td>
<td>Pubertal rat</td>
<td>In vivo</td>
<td>N/A</td>
<td>Dysfunction and morphological</td>
</tr>
</tbody>
</table>
### C) Topoisomerase Inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Cell Type</th>
<th>Culture Type</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoposide</td>
<td>1µM (48 hrs)</td>
<td>Rat SSCs (isolated from pnd7-8)</td>
<td><em>In vitro</em></td>
<td>↓SSC clusters.</td>
<td>Marcon <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>Etoposide</td>
<td>25µM or 100µM (24 hrs)</td>
<td>SCs from prepubertal rat (pnd19)</td>
<td><em>In vitro</em></td>
<td>N/A</td>
<td>Aslani <em>et al.</em>, 2017</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.2µg</td>
<td>Prepubertal rat (pnd21)</td>
<td><em>In vivo</em></td>
<td>↑CC9, 8 and 3 activation 24 hrs after treatment in spermatocytes. Protein and mRNA of p53 and Bcl2 altered.</td>
<td>Ortiz <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.05g/kg/day Total doses 0.01, 0.02 &amp; 0.04g/kg</td>
<td>Prepubertal rat (pnd25)</td>
<td><em>In vivo</em></td>
<td>Analysed pnd26 and pnd32. ↑apoptotic differentiated spermatogonia and primary spermatocytes except for</td>
<td>Stumpf <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Beudet al., 2017</td>
<td>N/A</td>
<td>GC-6 spermatogonial cell line (rat)</td>
<td><em>In vitro</em></td>
<td>DNA strand breaks. Cell death without activation of apoptosis (externalization of phosphatidylserine).</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.1µM (24-72 hrs)</td>
<td>GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)</td>
<td><em>In vitro</em></td>
<td>Time- and dose-dependent ↑ in cytotoxicity.</td>
<td>Time- and dose-dependent ↑ in cytotoxicity (Ser-W3 more sensitive). ↑Oxidative stress, nuclear 8-oxo-deoxyguanosine ↓ glutathione levels 6 hrs. Glutathione supplementation did not affect survival.</td>
</tr>
<tr>
<td>Marcon et al., 2010</td>
<td>N/A</td>
<td>SCs from prepubertal rat (pnd19)</td>
<td><em>In vitro</em></td>
<td>N/A</td>
<td>No impact on cell viability or CC3 activation.</td>
</tr>
<tr>
<td>Stumpp <em>et al.</em>, 2004</td>
<td>N/A</td>
<td>Prepubertal rat (pnd25)</td>
<td><em>In vivo</em></td>
<td>Analysed pnd26 and pnd32. ↑apoptotic differentiated spermatogonia and primary spermatocytes except for</td>
<td>N/A</td>
</tr>
<tr>
<td>Treatment</td>
<td>Concentration</td>
<td>Treatment Method</td>
<td>Treatment Duration</td>
<td>Response</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>------------------</td>
<td>--------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>0.04g/kg</strong></td>
<td>Prepubertal rat (pnd25)</td>
<td>In vivo</td>
<td></td>
<td>SC adluminal with chromatin clumps and vacuolization. ↓ transferrin production from pnd45 onwards.</td>
<td>Stumpp <em>et al.</em>, 2006, 2008</td>
</tr>
<tr>
<td><strong>0.002g/kg (30 days)</strong></td>
<td>Pubertal rat (pnd30)</td>
<td>In vivo</td>
<td>↓ GCs, ↑ cell death (chromatin condensation). Damage still apparent 113 days after treatment.</td>
<td>N/A</td>
<td>Freitas <em>et al.</em>, 2002</td>
</tr>
<tr>
<td><strong>0.01µM (48 hrs)</strong></td>
<td>C18-4 spermatogonial cells (mouse)</td>
<td>In vitro</td>
<td>No effect on levels of γH2AX levels in telomeres or telomere dysfunction.</td>
<td>N/A</td>
<td>Liu <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>Irinotecan (SN38 metabolite)</td>
<td>0.1 and 1µg/ml (24 hrs)</td>
<td>Prepubertal rat (pnd5)</td>
<td>In vitro</td>
<td>Targets the proliferating germ cell population.</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>D) Vinca Alkaloids &amp; Antitumor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleomycin</td>
<td>0.1µM (48 hrs)</td>
<td>Rat SSCs (isolated from pnd7-8)</td>
<td>In vitro</td>
<td>↓ cluster number and area in culture.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1.5µM (48 hrs)</td>
<td>C18-4 spermatogonial cells (mouse)</td>
<td>In vitro</td>
<td>↑ DNA damage in the telomeres, no impact on telomerase activity.</td>
<td>N/A</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>500ug/kg (alternate day for 20 dys)</td>
<td>Pubertal rats (pnd 40)</td>
<td>In vivo</td>
<td>↓ Leydig cell nuclear area ↓ 3β-Hydroxysteroid dehydrogenase</td>
<td>N/A</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.1µM (48 hrs)</td>
<td>Rat SSCs (isolated from pnd7-8)</td>
<td>In vitro</td>
<td>Targets SSC in a dose-dependent manner.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>0.5µg/ml (24 hrs)</td>
<td>SCs from prepubertal rat</td>
<td>In vitro</td>
<td>↓ transferrin production.</td>
<td>N/A</td>
</tr>
<tr>
<td>(pnd18)</td>
<td>0.1µM (24-72 hrs)</td>
<td>GC-6 spermatogonial cell line (rat)</td>
<td>In vitro</td>
<td>↓cell viability, ↑cell death differentiated spermatogonia dose- and time-dependent manner. No observable DNA damage but activation of apoptotic pathways.</td>
<td>N/A</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

**E) Combination Treatments**

- **Bleomycin, Cisplatin & Etoposide**
  - 0.1µM (48 hrs)
  - Rat SSCs (isolated from pnd7-8)
  - In vitro
  - Combination had no additional impact on cluster size/area of SSCs.
  - N/A
  - Marcon et al., 2010

- **Vincristine & Doxorubicin**
  - 0.01µM (24-72 hrs)
  - GC-6 spermatogonial cell line (rat)
  - In vitro
  - Combination ↑cell death of spermatogonia dose-dependent manner in comparison to individual treatment.
  - N/A
  - Beud et al., 2017

ABP; androgen binding protein CC; cleaved caspase, GC: germ cells, hrs; hours, LC; Leydig cells, PMC; peritubular myoid cells, pnd; postnatal day, wks; weeks, SC; Sertoli cells, SCO; Sertoli cell only tubules, SSCs: spermatogonial stem cells.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (Drug/Doses)</th>
<th>Animal Model/Age</th>
<th>In vivo or In vitro</th>
<th>Effect</th>
<th>Papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amifostine</td>
<td>0.2g/kg Doxorubicin 0.003g/kg</td>
<td>Prepubertal rat (pnd6)</td>
<td>In vivo</td>
<td>No protective effects.</td>
<td>Jahnukainen et al., 2001; Hou et al., 2005</td>
</tr>
<tr>
<td>Amifostine</td>
<td>0.4g/kg Cisplatin 0.005g/kg</td>
<td>Prepubertal rat (pnd30)</td>
<td>In vivo</td>
<td>Partial protection, ↓ seminiferous tubule area and ↑ apoptotic spermatogonia and primary spermatocytes comparable to control or amifostine treated.</td>
<td>Lirdi et al., 2008</td>
</tr>
<tr>
<td>Amifostine</td>
<td>0.4g/kg Doxorubicin 0.005g/kg</td>
<td>Prepubertal rat (pnd30)</td>
<td>In vivo</td>
<td>Partially protects. Did not protect against DNA damage and negatively impacted on embryo development and pregnancy outcome.</td>
<td>Vendramini et al., 2010, 2012</td>
</tr>
<tr>
<td>Amifostine</td>
<td>1µM Doxorubicin 0.01-1µM (24hrs)</td>
<td>GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)</td>
<td>In vitro</td>
<td>Pre-treatment for 24 hrs or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line.</td>
<td>Tremblay and Delbes, 2018</td>
</tr>
<tr>
<td>Cartinine</td>
<td>0.25g/kg Etoposide 0.04g/kg</td>
<td>Prepubertal rat (pnd25)</td>
<td>In vivo</td>
<td>Analysed pnd30, 64 &amp; 100. Partial protection, reduction in TUNEL+ cells.</td>
<td>Okada et al., 2009</td>
</tr>
<tr>
<td>Cartinine</td>
<td>0.25g/kg/day Doxorubicin 0.005g/kg</td>
<td>Prepubertal rat (pnd30)</td>
<td>In vivo</td>
<td>Analysed pnd64 &amp; 100. Partial protection, ↓ TUNEL+ cells and sperm DNA damage. ↑ acrosome integrity pre-treatment, no impact on sperm motility and mitochondrial activity. ↓ lipid peroxidation and nitric oxide. ↑ fertility index and implantation rate improved.</td>
<td>Cabral et al., 2014, 2018</td>
</tr>
<tr>
<td>Supplement</td>
<td>Concentration</td>
<td>Doxorubicin Concentration</td>
<td>Cell Line</td>
<td>Treatment Duration</td>
<td>Treatment Details</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------</td>
<td>---------------------------</td>
<td>----------------------------------------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cartinine</td>
<td>10mM</td>
<td>0.01-1µM (24hrs)</td>
<td>GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)</td>
<td>In vitro</td>
<td>Pre-treatment for 24 hrs or co-treatment had no impact on cytotoxicity in the Ser-W3 cell line.</td>
</tr>
<tr>
<td>Curcumin</td>
<td>5µM</td>
<td>0.01-1µM (24hrs)</td>
<td>GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)</td>
<td>In vitro</td>
<td>Pre-treatment for 24 hrs or co-treatment no impact on cytotoxicity in the Ser-W3 cell line.</td>
</tr>
<tr>
<td>Ginseng Intestinal</td>
<td>0.05g/kg/day</td>
<td>0.05g/kg per day</td>
<td>Prepubertal rat (pnd28)</td>
<td>In vivo</td>
<td>Partially protects.</td>
</tr>
<tr>
<td>Metabolite I (GIM-I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>40µg/ml</td>
<td>0.01-1µM (24hrs)</td>
<td>GC-6 spermatogonial and Ser-W3 immature SC cell line (rat)</td>
<td>In vitro</td>
<td>Pre-treatment for 24 hrs or co-treatment no impact on cytotoxicity in the Ser-W3 cell line.</td>
</tr>
</tbody>
</table>

GC; germ cells, SC; Sertoli cells, SSCs; spermatogonial stem cells
Records identified through database searching (n = 942) → Additional records identified through other sources (n = 21) → Records after duplicates removed (n = 535) → Records screened (n = 535) → Full-text articles assessed for eligibility (n = 68) → Studies included in qualitative synthesis (n = 51) → Records excluded (n = 467) → Full-text articles excluded, with reasons (n = 17)
- Review paper (n=1)
- Does not include histological analysis of the testis (n=7)
- Tissues/cells are pubertal/adult (n=5)
- Exposure to chemotherapy agents in utero (n=1)
- Doses of individual chemotherapy agents not included (n=3)

Figure 1

254x190mm (72 x 72 DPI)
Figure 2

187x232mm (96 x 96 DPI)