Toxicology Reports 8 (2021) 114–123

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Chemotherapy induced damage to spermatogonial stem cells in prepubertal mouse in vitro impairs long-term spermatogenesis

Federica Lopes [a,b,*], Prathima Tholeti [a,c], Satish K. Adiga [c], Richard A. Anderson [b], Rod T. Mitchell [b,**,1], Norah Spears [a,**,1]

[a] Biomedical Sciences, University of Edinburgh, Edinburgh, UK
[b] MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, UK
[c] Department of Clinical Embryology, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal, India

ARTICLE INFO

Edited by: DR. A.M. Tsatsaka

Keywords:
Chemotherapy
In vitro
Spermatogonial stem cells
Testis
Prepubertal mouse

ABSTRACT

Chemotherapy can affect testis development of young boys with cancer, reducing the chances of fatherhood in adulthood. Studies using experimental models are needed to determine the damage caused by individual chemotherapy drugs in order to predict the risk of infertility and direct patients towards appropriate fertility preservation options. Here, we investigated the individual role of two drugs, cisplatin and doxorubicin, using an *in vitro* culture model of prepubertal (postnatal day 5) mouse testis that supports induction and maintenance of full spermatogenesis. Twenty-four hour exposure with either drug at clinically-relevant doses (0.25, 0.5 or 0.75 μg/mL for cisplatin, or 0.01, 0.03 or 0.05 μg/mL for doxorubicin), induced an acute significant loss of spermatogonial stem cells (SSCs; $PLZF^+$), proliferating SSCs ($PLZF^+BrdU^+$), total germ cells (MVH^+), and spermatocytes ($SCP3^+$) one week after chemotherapy exposure. By the time of the first (Week 4) and second (Week 8) waves of spermatogenesis, there was no longer any effect on SSC or proliferating SSC numbers in drug-exposed testis compared to untreated tissue: however, the populations of total germ cells and spermatocytes were still lower in the higher-dose cisplatin treated groups, along with a reduced frequency of round and elongated spermatids in both cisplatin- and doxorubicin-treated testis fragments. Overall, this study details a direct impairment of germ cell development following acute chemotherapy-induced damage during the prepubertal phase, most likely due to an effect on SSCs, using an *in vitro* culture system that successfully recapitulates key events of mouse spermatogenesis.

1. Introduction

Improvements in treatment and diagnosis have dramatically increased life expectancy for children with cancer, with more than 80 % 5-year survival rates reported in those diagnosed before age 14 [1–3]. This medical success has resulted in a growing focus on the quality of life of childhood cancer survivors, with increasing importance given to reducing the long-term side effects of anti-cancer therapy. One of the most important long-term impacts of childhood cancer treatment is on fertility [4], and although discussions of fertility can be challenging for children and parents/carers, the possibility of loss of fertility generates great concern amongst cancer survivors [5,6].

Chemotherapy can affect testicular development, resulting in impaired spermatogenesis and thus infertility in adulthood [7]; however, in many cases it remains problematic to predict the likelihood of an individual developing fertility problems. Furthermore, post-treatment assessment of the severity of any damage is not possible during childhood due to a lack of clinical biomarkers. For the prepubertal boys who are most at risk, there is a lack of fertility preservation options available. It is not possible to preserve fertility through semen cryopreservation, since fully mature sperm cells are not yet produced. In time, it may be possible to restore fertility using testicular biopsies collected from children with cancer before the onset of any potentially sterilising treatment [8]. An alternative solution is to develop preventive measures that will reduce chemotherapy-induced damage to the reproductive organs [9–12]. In order for any such protective agent to be identified, we

* Corresponding author at: MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, UK.
** Corresponding authors.
E-mail addresses: Federica.Lopes@ed.ac.uk (F. Lopes), Rod.Mitchell@ed.ac.uk (R.T. Mitchell), Norah.Spears@ed.ac.uk (N. Spears).
[1] Equal contribution.

https://doi.org/10.1016/j.toxrep.2020.12.023
Received 11 August 2020; Received in revised form 24 November 2020; Accepted 21 December 2020
Available online 26 December 2020

require more understanding about the mechanism of action and long-term consequences of chemotherapy agents on the developing testis. Currently, the degree of chemotherapy-induced damage to the testis remains unpredictable, especially in children. It will vary according to the therapeutic regimen (type of anticancer agents, dose, and timing), as well as the patient's age, and individual sensitivity [13]. Clinical studies provide limited information in regard to the risk of infertility posed by each chemotherapy compound, since these are almost always administered in combinations. Nonetheless, some classes of drugs, such as alkylating agents, have been associated with a high risk of testicular damage [14]. Cisplatin is an alkylating-like platinum-based compound that is often used to treat solid tumours in paediatric cancer patients [15,16]. The mechanism of action of cisplatin in cancer treatment relies on the formation of DNA adducts that induce cell death [17]. Doxorubicin is an anthracycline antibiotic used to treat a range of childhood cancers, such as lymphoma, sarcoma, neuroblastoma and Wilms tumour [18]. Inhibition of Topoisomerase II is the main mechanism of action here, in turn inducing accumulation of DNA fragments during replication and ultimately cell death [17]. Both drugs are considered to have a medium (doxorubicin)-to-high (cisplatin) risk of producing testicular damage in humans [1,19], and both have been associated with acute germ cell loss in a mouse model [20]. In the longer term, however, it is vital to understand the potential for recovery of spermatogenesis following any chemotherapy insult, with any such recovery dependant on the survival and/or repopulation of spermatogonial stem cells (SSCs).

Spermatogenesis relies on self-renewal of SSCs alongside continuous production of differentiating germ cells from SSCs to generate sperm. Spermatogenesis is dependent on a local somatic cell 'niche' environment. The characterization of the detrimental effect of chemotherapy drugs upon specific cell types within the testicular tissue (*e.g.* germ and/or somatic cells) is key for accurate prediction of the risk to fertility, and for the development of targeted protective strategies. Direct insult of chemotherapy drugs on germ cells has been shown in animal studies [21,22] and confirmed in the analysis of childhood testicular biopsies [14,23]; this could well be a leading cause of oligozoospermia or azoospermia in adulthood [24] and of the reduced chances of paternity that has been demonstrated in a long-term cohort study of childhood cancer survivors [4].

In the present study, we have used an *in vitro* culture system that supports *ex vivo* spermatogenesis in prepubertal mouse testis, as first developed by Sato et al. [25], in order to examine the potential of the testis to recover from chemotherapy-induced injury. Here, the culture method has been amended to improve tissue quality and consistency, to better support toxicological studies such as this. We have used this approach to assess the impact of patient-relevant concentrations of the chemotherapy agents cisplatin and doxorubicin on the immature testis, with a focus on SSC survival, proliferation and differentiation, and on subsequent spermatogenesis.

2. Material and methods

2.1. Animals

CD-1 mice were housed in a 14h:10 h light:dark cycle, with food and water provided *ad libitum*. All experiments were approved by the Animal Welfare and Ethical Review Board (AWERB) at the University of Edinburgh and all procedures were carried out in accordance with UK Home Office Regulations.

2.2. Testicular testis culture

Mouse testes (n = 28) were obtained from 14 pups on postnatal day (pnd) 5 and placed into Leibovitz L-15 medium (Invitrogen), supplemented with 3 mg/mL bovine serum albumin (BSA; Sigma-Aldrich). Culture (n = 5-7 runs) was performed in serum-free medium, as previously published [20,26], with minor modifications. In brief, after removal of tunica albuginea, each testis was fragmented into 0.5−1 mm^3 cubes, with the tissue fragments then randomly distributed in a 24-well plate, each fragment in an individual well. A liquid-air interphase culture was set up by placing each testis fragment onto a polyacrylamide membrane (Whatman nucleopore, Camlab) floating on 1 mL of culture medium composed of αMEM medium (Invitrogen) with 10 % knockout serum replacement (Invitrogen) and 1% antibiotic-antimycotic (Thermofisher) at 34 °C in 5% CO_2 humidified atmosphere. After a 24 h resting period (Day 1), testis fragments were exposed to chemotherapy drugs added to the culture medium in a range of concentrations as detailed below (Day 2). On Day 3, tissues were moved into drug-free medium and maintained in that environment for the remainder of the culture period, supplemented with fresh medium on alternate days. Twenty-four hours before the end of culture period, 1.5 μg/mL of 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was added to the medium to allow subsequent comparison of cell proliferation rates. Tissues were processed for analysis at three different time points post drug exposure: 7 days (Week 1), 28 days (Week 4) and 56 days (Week 8). Age-matched control testes were collected from one-two mice at pnd 13 for Week 1, pnd 33 for Week 4, and pnd 61 for Week 8, to enable comparative analysis of organ fragment developed *in vitro versus* an age-equivalent *in vivo* tissue. Mouse spermatogenesis requires an average of 34.5 days to be completed [27]. The onset of puberty in mice ranges between 30–40 pnd, with the first round and elongated spermatids appearing at 20–30 pnd [28].

2.3. Chemotherapy agents

Cisplatin and doxorubicin (Sigma-Aldrich) were dissolved in sterile water and added to the culture medium to reach final concentrations of 0.25, 0.5 or 0.75 μg/mL for cisplatin, and 0.01, 0.03 or 0.05 μg/mL for doxorubicin. Control media was supplemented with vehicle (water, 0.1 %). Drug concentrations were selected to be at the lower end of those reported in the serum of cancer patients: for cisplatin 0.05−4 μg/mL [29–32]; for doxorubicin 0.02−0.3 μg/mL [33,34].

2.4. Histology and immunohistochemistry

At the end of the culture period, tissues were fixed for 1 h in either Bouin's solution (Sigma-Aldrich) or 4% Neutral Buffered Formalin (NBF; Sigma-Aldrich). Serial sections (5 μm) were cut until the entire wax-embedded tissue block was sectioned, with the complete set of sections from Bouin's-fixed fragment used for histological examination following staining with haematoxylin and eosin (H&E) or Period acid-Schiff and haematoxylin (PAS), and representative cross-sections of the NBF-fixed fragments used for subsequent immunohistochemistry (IHC). Testes collected for age-equivalent (pnd 13 - Week 1, pnd 33 - Week 4, and pnd 61 - Week 8) *in vivo* analysis were fixed overnight in Bouin's solution, sectioned (5 μm), with randomly selected sections stained with H&E or PAS.

2.5. Morphological analysis

Every H&E stained serial section per testicular fragment was examined, and representative photomicrographs taken by blind-to-treatment assessor (DMLB Leica microscope, Leica Microsystem Ltd.). Morphological identification of undifferentiated germ cells and somatic cells was carried out according to previously published criteria [35]. Lining the basal membrane, gonocytes were identifiable by their large round nuclei and neat cellular borders, while undifferentiated spermatogonia were identified by their typical oval nuclei with few nucleoli. In a luminal position, round spermatids (RS) were identified by their smaller size compared to germ cells at other developmental stages (spermatogonia and spermatocytes) and/or by a small dark area beside the nucleus indicating the developing acrosome. Randomly selected sections

representative of each experimental group were PAS stained to confirm the presence of the acrosome for RS identification. Elongated spermatids (ES) were identified by a compact dense structure representing the precursor for the hook-shaped head of the mature spermatozoa. The presence of haploid male germ cells (round or elongated spermatids) was recorded for each individual experimental tissue fragment. Sections used for IHC, were also analysed to assess the presence of ES, whose typical morphology (described above) was further identified by DNA counterstaining (see IHC section).

2.6. Immunohistochemistry

One or two non-consecutive sections representative of the centre (core) of each testis fragment were used for each immunostaining run. IHC was performed as in Smart et al. [20]. In brief, after heat mediated antigen retrieval in 10 mM citrate buffer (pH6; Fisher Chemical), a solution of 20 % goat serum with 0.1 % Triton X-100 (Sigma-Aldrich) and 5 % BSA (Sigma-Aldrich) in PBS was used for blocking for 1 h at room temperature (RT). Washes in phosphate-buffered saline (Fisher Scientific UK Ltd.) with 0.1 % Triton X-100 were done in between each step. Primary antibody (Table 1) was applied overnight at 4 °C in a humidified chamber, followed by 1 h incubation at RT with appropriate secondary antibody (Table 1). Omission of primary or secondary antibody was used to assess specificity of IHC reactions. Sections were counterstained with DAPI (4,6-Diamidino-2phenylindole; 1:5000; Invitrogen), then mounted with Vectashield hard-mounting medium (Vector Laboratories).

Images were collected (Leica DM5500B microscope with DFC360FX camera, Leica Microsystem Ltd.) and analysed by blind-to-treatment assessor (cell counter plugin, ImageJ/Fiji software). For the first set of IHC (double IHC #1, Table 1), the number of $PLZF^+$ (SSCs), $BrdU^+$ (proliferating cells) and double $PLZF^+BrdU^+$ (proliferating SSCs) cells was manually counted. For the second set of IHC (double IHC #2, Table 1), the number of MVH^+ (germ cells) and $SCP3^+$ (spermatocytes) was manually counted. Density was calculated as the total number of each cell type relative to the total area of seminiferous tubules (mm^2) per section.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software, to determine statistical significance of treatment groups compared with control tissue. For results obtained from IHC, Kolmogorov-Smirnov normality test was used to assess Gaussian distributions of data. One-way ANOVA test was used for normally distributed data, followed by Bonferroni's multiple comparison. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used where normality could not be shown. The frequency of RS present in Week 4 and ES in Week 8 in treated tissues was compared with frequency of RS and ES in relative control tissues. Data were considered statistically significant when p value was ≤ 0.05.

3. Results

3.1. Optimization of in vitro culture system of prepubertal mouse testis supporting initiation and maintenance of spermatogenesis for toxicological investigations

Testicular tissue fragments isolated from prepubertal (pnd 5) mice and maintained in culture for up to eight weeks were able to support full spermatogenesis *in vitro*. Progression of spermatogenesis and tissue morphology were assessed using H&E stained histological sections and IHC (Fig. 1). The culture system successfully supported initiation and progression of spermatogenesis, with germ cell differentiation from gonocytes in pre-culture tissue (Fig. 1A), to elongated spermatids (ES) after eight weeks in culture (Fig. 1B). At the end of eight weeks in culture, the architecture of the testis was well preserved, with seminiferous tubule integrity conserved. There was also a notable absence of necrosis or disorganization at the centre of the tissue, indicating good survival of the entire tissue piece (Fig. 1B). Across the period of tissue culture, the organ culture system sustained normal testicular development, matching *in vivo* observations. After one week of culture, tubules contained spermatogonia, as well as differentiating germ cells entering the meiotic phase (spermatocyte - leptotene) populating the lumen of the seminiferous tubules (Fig. 1C): this was similar to that seen in age-matched control tissues obtained from pnd 13 mice (Fig. 1D). At Week 4 of culture, germ cell differentiation had advanced in many seminiferous tubules, with some germ cells now at the spermatocyte (pachytene) stage, and others that had progressed to haploid germ cells (RS; Fig. 1E): a similar pattern was observed in age-matched control tissues collected from pnd 33 mice (Fig. 1F). At the end of culture, at Week 8, there were also ES present in a few tubules (Fig. 1G). ES were also evident in age-matched tissues collected from pnd 61 mice (Fig. 1H). Germ cells at different developmental phases were also observed in detail in IHC stained sections (Fig. 1I), with the identification of germ cells (MVH^+ cells) during meiotic phases [prophase I: pachytene (Fig. 1I-a) and diplotene (Fig. 1I-b) spermatocytes; telophase I spermatocytes (Fig. 1I-c)] and post-meiotic, with ES identifiable by the characteristic shape, with a typical condensed nucleus resembling the head of spermatozoa and absence of MVH expression (Fig. 1I-d). It was notable that heterogeneity in tubule morphology increased as the culture proceeded: while many tubules supported active spermatogenesis and were packed with germ cells at different phases of differentiation as described, other tubules appeared to be enlarged, while tending to have a reduced germ cell population (Fig. 1B).

3.2. Cisplatin and doxorubicin lead to a loss of SSCs in the short-term only

The optimised culture system was used to assess the reproductive toxicity of two chemotherapy drugs commonly used in the treatment of childhood cancer patients, cisplatin and doxorubicin. Testicular organ fragments from prepubertal mice were exposed *in vitro* to patient-relevant concentrations of cisplatin (0.25, 0.5 or 0.75 μg/mL) or doxorubicin (0.01, 0.03 or 0.05 μg/mL), after which IHC was carried out to examine the density of SSCs, the density of all proliferating cells and the

Table 1
Antibodies and conditions for immunohistochemistry.

	Primary Ab[a]	Marker	Cat. N#	Specie	Dilution	Secondary Ab[b]	Cat. N#
Double IHC #1	anti-PLZF	SSCs	ab189849	rabbit	1:3000	Alexa Fluor 488 anti-rabbit	A11011
	anti-BrdU	proliferative cells	ab6326	rat	1:500	Alexa Fluor 568 anti-rat	A-11031
Double IHC #2	anti-MVH/DDX4	germ cells	ab13840	rabbit	1:200	Alexa Fluor 488 anti-rabbit	A11011
	anti-SCP3	spermatocytes	ab97672	mouse	1:500	Alexa Fluor 568 anti-mouse	A-21124

[a] All primary antibodies were purchased from Abcam.
[b] All secondary antibodies were raised in goat, purchased from Invitrogen and used at 1:200 dilution. Abbreviations: PLZF, Promyelocytic Leukaemia Zin Finger protein; BrdU, 5-bromo-2'-deoxyuridine; MVH/DDX4, Mouse Vasa Homologue/DEAD-Box Helicase 4; SCP3, Synaptonemal Complex Protein 3.

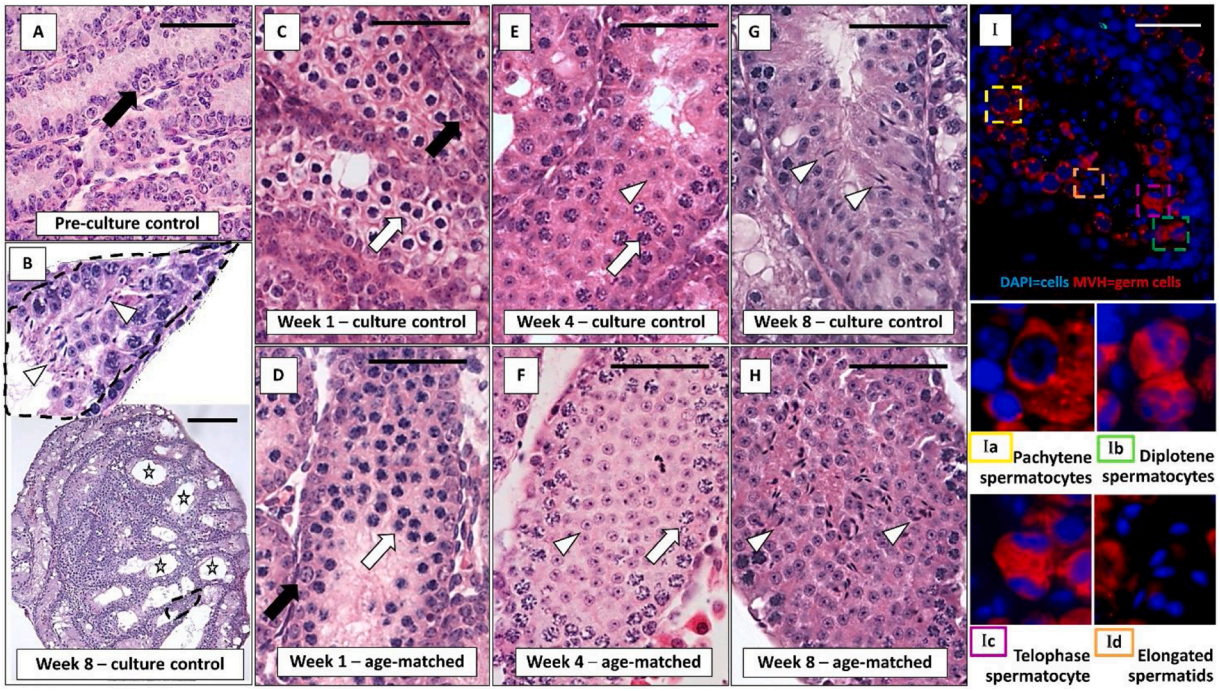


Fig. 1. Induction and progression of spermatogenesis over eight weeks in an *in vitro* model of mouse prepubertal testis development. Representative photomicrographs of haematoxylin & eosin and immunohistochemically stained sections of mouse testis. (**A**) Post-natal day (pnd) 5 mouse testis (pre-culture control), containing gonocyte/undifferentiated spermatogonia (black arrow). (**B**) Week 8 culture control testis, containing elongated spermatids (ES; white arrowhead), visible in the magnified image of one tubule, were the most differentiated germ cells seen in cultured tissue. No sign of necrosis can be observed in the centre of the tissue, with many seminiferous tubules containing active spermatogenesis and some enlarged tubules (star). (**C**) At Week 1 of culture, testis contains germ cells entering meiosis (primary spermatocyte – white arrow), along with spermatogonia (black arrow), resembling testis morphology of (**D**) pnd 13 mouse testis. (**E**) At Week 4 of culture, testis contains germ cells in pachytene phase of meiosis (white arrow) and post-meiosis (round spermatids – white arrowhead), resembling testis morphology of (**F**) pnd 33 mouse testis. (**G**) At Week 8 of culture, testis contains ESs (white arrowhead), as in (**H**) testis from pnd 61 mouse. (**I**) Different developmental phases of spermatogenesis identified by characteristic changes in nuclear morphology after immunostaining for MVH^+ cells (red), counterstained with DAPI (blue) for cell nuclei, showing spermatocytes in prophase: (**I-a**) pachytene (yellow inset) or (**I-b**) diplotene (green inset); (**I-c**) spermatocytes in telophase (purple inset); and (**I-d**) MVH^- elongated spermatids (orange inset). Scale bar in A represents 50 μm; in B, 100 μm; in C – H, 25 μm; in I, 50 μm.

density of proliferating SSCs (Fig. 2, A–C respectively). Drug treatment was administered for 24 h only, on Day 2 of culture, with tissues then maintained for up to eight weeks in drug-free culture medium. Three time points were selected to assess the impact of chemotherapy exposure: short- (Week 1), mid- (Week 4), and long-term (Week 8). To investigate the acute effect of chemotherapy exposure on the SSC population, quantification was carried out to determine the density of $PLFZ^+$ cells at Week 1. SSC density was significantly reduced in tissue exposed to all three cisplatin concentrations and to the two higher doxorubicin concentrations compared with control tissues ($p \leq 0.001$; Fig. 2D). To verify whether such short term loss was due to interference with cellular proliferation, BrdU incorporation by cells within the tubules was determined: a similar loss of proliferating cells within the seminiferous tubules was observed in testis fragments exposed to all cisplatin or doxorubicin concentrations ($p \leq 0.001$; Fig. 2E). Therefore, we looked specifically at the proliferating SSC population ($PLFZ^+BrdU^+$). This was also reduced after exposure to the two highest cisplatin concentrations ($p \leq 0.05$ for 0.5 μg/mL cisplatin; $p \leq 0.001$ for 0.75 μg/mL cisplatin) and to the highest doxorubicin concentration ($p \leq 0.05$ for 0.05 μg/mL doxorubicin) (Fig. 2F). We then determined whether such chemotherapy induced damage persisted four weeks later, when the first, usually not fertile, wave of spermatogenesis had likely taken place. At Week 4 after chemotherapy exposure, the negative impact on germ cells was maintained only for the highest concentration of doxorubicin, which resulted in a significant reduction in density of SSCs ($p \leq 0.05$ for density of $PLZF^+$; Fig. 2G), proliferating cells ($p \leq 0.01$ for $BrdU^+$; Fig. 2H) and proliferating SSCs ($PLZF^+BrdU^+$; Fig. 2I) compared to control. At Week 8, there was no longer any significant effect of chemotherapy exposure on either SSC or proliferating SSC populations (Fig. 2J–L). Despite the significant loss of number of SSCs and proliferating SSCs, the proportion of SSCs that were proliferating during the last 24 h of each culture end points did not change over time and across drug treatments when compared to control (Supplementary Fig. 1).

3.3. *Differing effects of cisplatin and doxorubicin on meiotic progression during prepubertal mouse spermatogenesis*

To determine whether the effects of cisplatin or doxorubicin on the testicular stem cell population resulted in changes in the ability of germ cells to progress through meiosis, MVH^+ germ cells (Fig. 3A) and $SCP3^+$ meiotic cells (Fig. 3B) were quantified at all three time points and their density expressed as relative to area (mm^2) of seminiferous tubules. At Week 1, all drug treatments resulted in a significant loss of total germ cells compared to control cultures ($p \leq 0.001$; Fig. 3C). In contrast, the density of spermatocytes was significantly reduced by all cisplatin concentrations ($p \leq 0.001$) but by only the highest concentration of doxorubicin ($p \leq 0.05$; Fig. 3D). At Week 4, there was significant reduction in total germ cells at the highest concentrations of cisplatin or doxorubicin (both $p \leq 0.05$; Fig. 3E); there was no longer any effect of cisplatin on spermatocyte density, but this did remain significantly lower after exposure to the top two doses of doxorubicin (mid dose $p \leq 0.05$; high dose $p \leq 0.01$; Fig. 3F). At Week 8, cisplatin-exposed testis fragments showed a significant loss of total germ cells and of spermatocytes at the mid- and high-concentrations ($p \leq 0.05$ for all); in contrast, there was no longer any significant effect of doxorubicin-exposure (Fig. 3G, H).

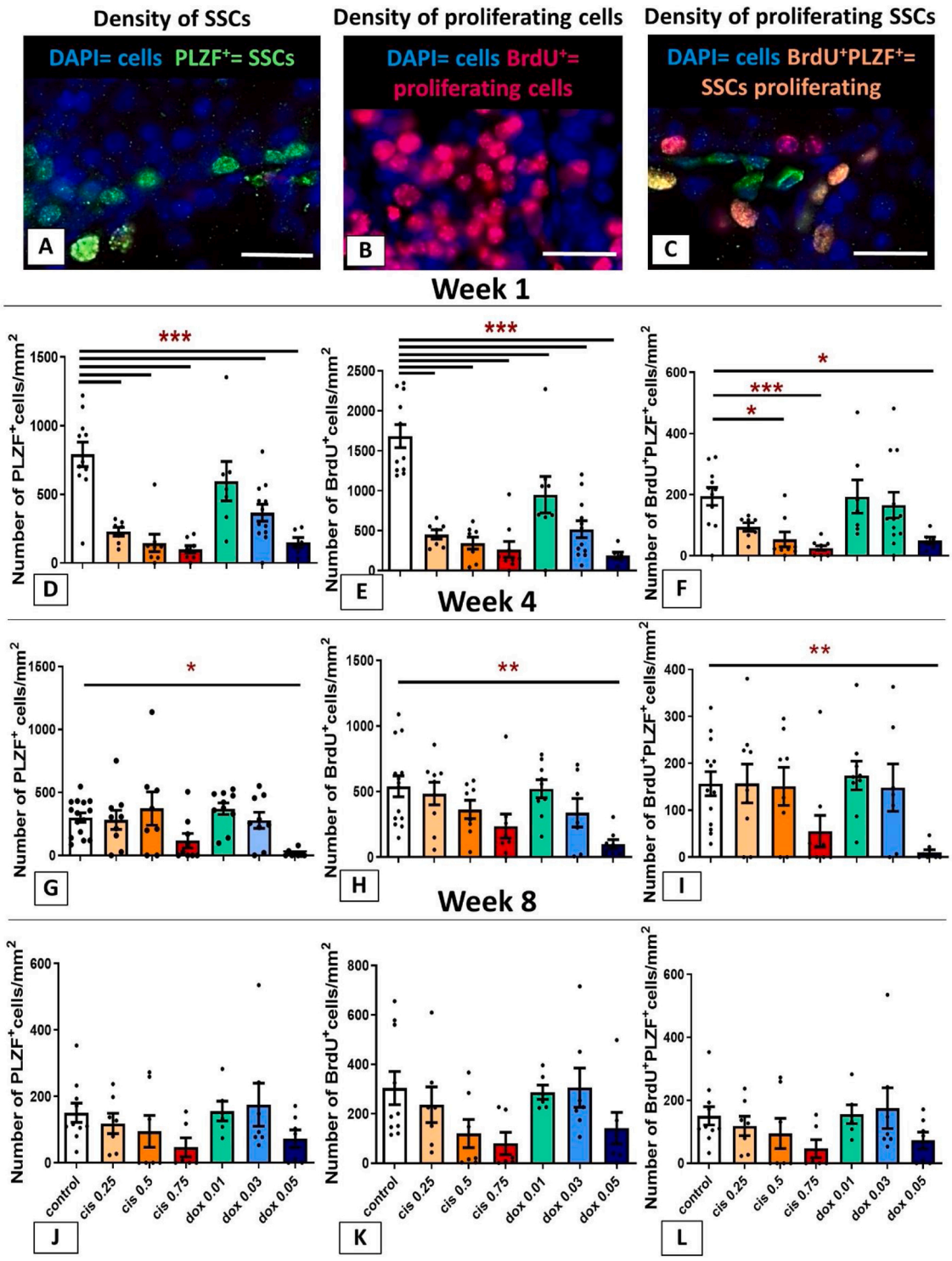
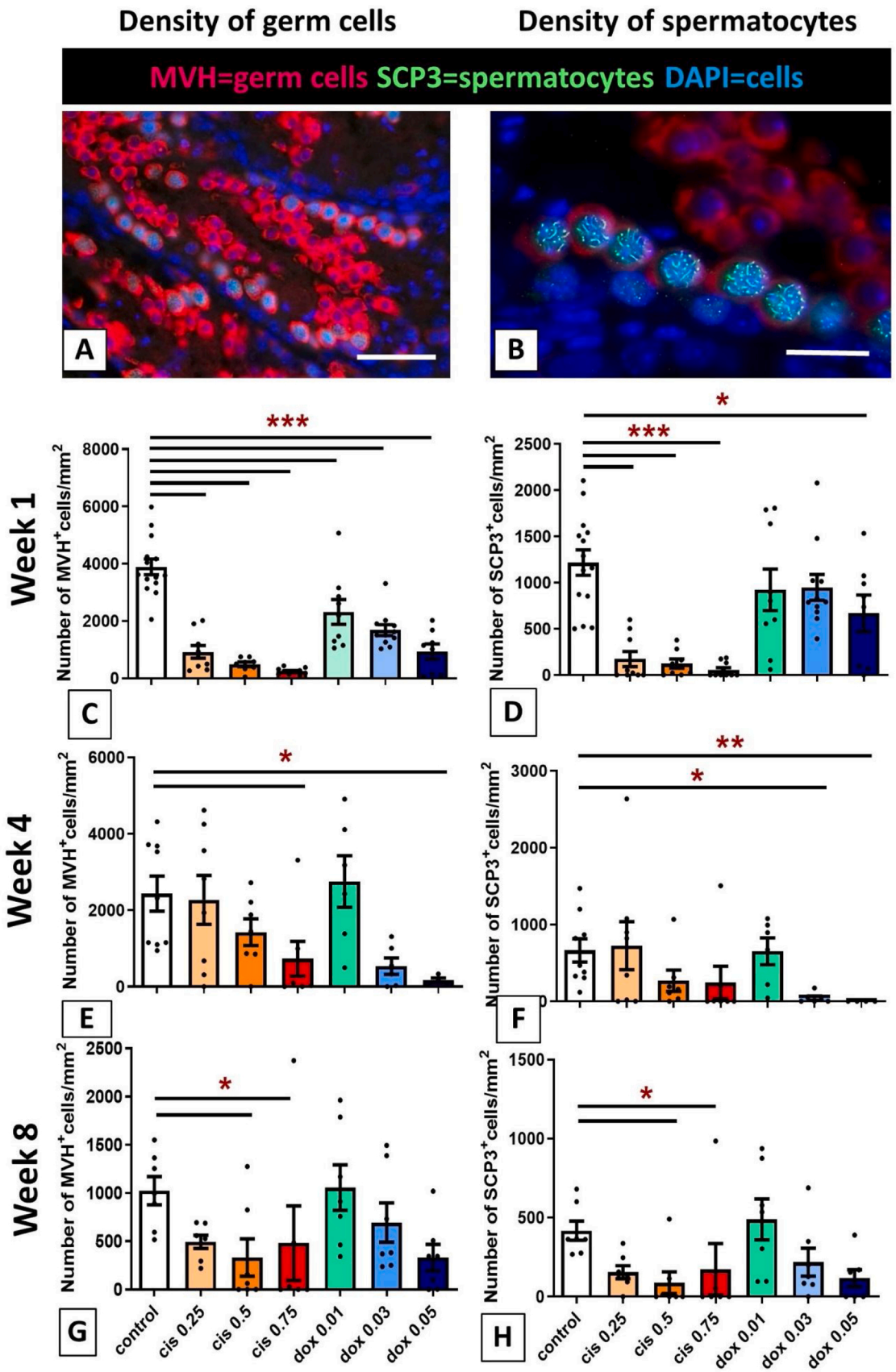


Fig. 2. Effect of cisplatin or doxorubicin on spermatogonial stem cells, proliferating cells within seminiferous tubules and proliferating spermatogonial stem cells in mouse testis exposed *in vitro*. Representative photomicrographs of immunohistochemistry to identify (**A**) spermatogonial stem cells (SSCs; $PLZF^+$; green); (**B**) proliferating cells ($BrdU^+$; red); (**C**) proliferating SSCs (double stained $PLZF^+BrdU^+$; orange-yellow); cell nuclei counterstained with DAPI (blue). Scale bars represent 25 μm. (**D-L**) Graphs showing cell quantification expressed per seminiferous tubule area (mm^2) in mouse testis fragment after chemotherapy drugs exposure at (**D-F**) Week 1; (**G-I**) Week 4; and (**J-L**) Week 8 time points. There was a significant reduction in cell densities one week after cisplatin or doxorubicin exposure, not persisting at Week 4 (apart at the highest doxorubicin concentration) or Week 8. Values shown are means ± SEM, with each data point representing an individual organ fragment (n = 6-14). Statistical analysis compared treated *versus* control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4. The presence of round and elongated spermatids after exposure to cisplatin or doxorubicin

Histological sections of cultured tissues were examined at Weeks 4 and 8 to determine whether the short-term chemotherapy-induced loss of SSCs impacted on the later stages of germ cell development. The number of testicular tissues in which RS (Fig. 4A and Supplementary Fig. 2) or ES (Fig. 4B) were present was recorded. In total, 54 % of



(caption on next page)

Fig. 3. Effect of cisplatin or doxorubicin on spermatogonial germ cells and spermatocytes in mouse testis exposed *in vitro*. Representative photomicrographs of immunohistochemistry for the identification of (**A**) germ cells (MVH^+; red); and (**B**) spermatocytes ($SCP3^+$; green); cell nuclei counterstained with DAPI (blue). Scale bar represents 50 μm in **A**; 40 μm in **B**. (**C-H**) Graphs showing cell quantification expressed per seminiferous tubules area (mm^2) in mouse testis fragment after chemotherapy drug exposure at (**C-D**) Week 1; (**E-F**) Week 4; and (**G-H**) Week 8 time points. (**C**) At Week 1, significant loss of germ cells was seen after both cisplatin and doxorubicin exposure. (**E**) Such reduction remained significant at the highest drug concentrations at Week 4 and (**G**) at the two highest cisplatin concentrations at Week 8. (**D**) Spermatocytes were significantly reduced in all cisplatin concentrations and at the highest doxorubicin concentration at Week 1. (**F**) At Week 4, the loss was significant in the two highest doxorubicin exposed tissues; and (**H**) at Week 8, at the two highest cisplatin concentrations. Values shown are means ± SEM, with each data point representing an individual organ fragment (n = 6-14). Statistical analysis compared treated *versus* control group. *p < 0.05, **p < 0.01, ***p < 0.001.

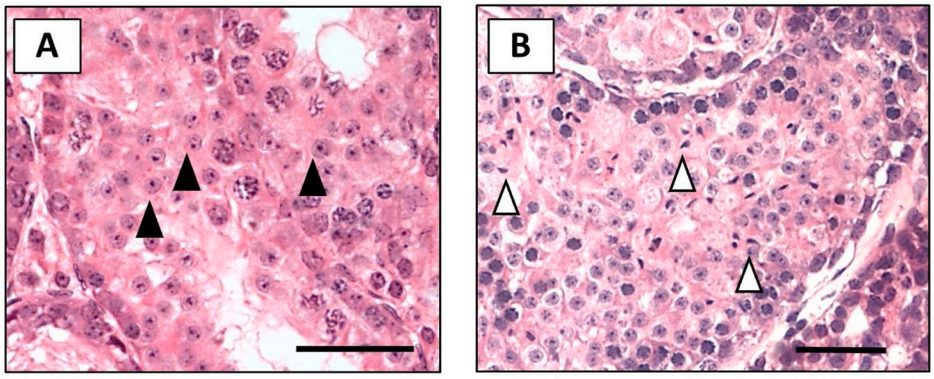


Fig. 4. Morphological evaluation of testicular fragments with spermatogenesis 4 and 8 weeks after cisplatin or doxorubicin exposure. Representative photomicrographs of histological sections of mouse testis at (**A**) Week 4 and (**B**) Week 8. Scale bars represent 25 μm. Round spermatids (RS; black arrowhead) and elongated spermatids (ES; white arrowheads) were identified as endpoint spermatogenesis stage for Weeks 4 and 8, respectively. (**C**) Quantification of testicular fragments with tubules with RS or ES, given relative to the number of fragments analysed.

C

Experimental group	Week 4 - Number of testis fragments with round spermatids/number of testis fragments analysed (%)	Week 8 - Number of testis fragments with elongated spermatids/number of testis fragments analysed (%)
control	6/11 (54)	6/14 (42)
cis 0.25	2/8 (25)	2/10 (20)
cis 0.5	0/6 (0)	3/9 (33)
cis 0.75	1/8 (12.5)	1/9 (11)
dox 0.01	5/8 (62.5)	1/7 (14)
dox 0.03	0/8 (0)	1/10 (10)
dox 0.05	0/5 (0)	0/11 (0)

control testis fragments maintained in culture for four weeks contained RS. The percentage of fragments with RS was consistently lower (0–25 %) in cisplatin-exposed tissues, while RS were not seen in any tissue exposed to the two highest doxorubicin concentrations (Fig. 4C). Similarly, two months after chemotherapy exposure, spermatogenesis proceeded to the ES phase in around 40 % of control fragments, with lower frequency found in cisplatin- (11–33 %) or doxorubicin- (0–14 %) exposed tissues (Fig. 4C).

4. Discussion

This study assessed the impact of patient-relevant concentrations of cisplatin and doxorubicin, two drugs often included in protocols for oncologic paediatric patients, on the development of the immature testis. The treatment design aimed to evaluate the histopathological changes of prepubertal mouse testis caused by *in vitro* exposure to chemotherapy drugs, and also to assess the long-term consequences of acute SSC loss on subsequent self-renewal and differentiation. Both drugs induced acute loss of SSCs that was not maintained two months later; however, high concentrations of cisplatin impaired germ cell development in the longer term.

Both chemotherapy agents resulted in a short-term fall in SSCs and total germ cells, confirming previous work from our group [20,36], as well as others also using a prepubertal rodent model [15,37]. To date, no studies are available on the direct acute impact of cisplatin or doxorubicin on the SSC/germ cell pool in children, due to the scarcity of testicular samples collected before and after chemotherapy. Also, the frequent administration of drugs in combination renders it challenging to identify the mechanism of action of individual agents on a specific cell population within the human testis.

It is likely that most chemotherapy agents, including cisplatin and doxorubicin, act by interfering with the proliferative process and/or by inducing apoptosis in germ cells, as they do in neoplastic cells [38,39]. Here, the number of proliferating germ cells within the seminiferous tubules was drastically reduced one week after cisplatin or doxorubicin exposure: crucially, this included a significant reduction in the number of proliferating SSCs. We have previously shown increased expression of apoptotic markers in spermatogonial gem cells before the number of such cells decline [20], with others showing evidence of apoptotic pathways activation [15,40,41]. Indeed, germ cell damage can impact negatively on the supporting somatic Sertoli cells, exacerbating the fertility impairment [42]. Further studies will be required to translate animal findings to effects on prepubertal boys; understanding the specific impact and mechanism of actions of individual anticancer agents, such as cisplatin and doxorubicin, on the key pool of SSCs will help to predict the long-term risk of infertility during adulthood.

Here, testis fragments were kept in culture for up to two months following chemotherapy exposure, to assess whether the acute damage to SSCs would persist, and whether there would be any effect on the later phases of spermatogenesis. In the longer term, the difference in SSC density between control and drug-exposed testes dissipated, as did the difference in number of SSCs that were proliferating during the last 24 h of the culture period. It has been suggested that a gonadotoxic insult would increase the mitotic rate of undifferentiated spermatogonial cells [43], in order to repopulate the seminiferous epithelium; an increased mitotic activity could in turn predispose the germ cells to more severe damage during any subsequent cycles of treatment [44]. Here, no change was noted in the proportion of SSCs that were proliferating over time or across treatments, however it is possible that the time points selected did not pick up a change occurring at other times. Furthermore, SSCs could increase their number by selectively opting for self-renewal over differentiation, without necessarily modifying their proliferation rate.

Adult spermatogenesis is quantitatively dependent on the number of SSCs and of their supporting Sertoli cells. A strong correlation between the number of SSCs remaining following chemotherapy and the recovery time for spermatogenesis has been demonstrated in adult mice [45]. In the present study, germ cell density remained low two months after cisplatin but not doxorubicin exposure (although a non-statistically significant dose-response trend was present). A limited number of studies have investigated the impact on SSCs of chemotherapy administered to prepubertal animals using *in vivo* models [43], with subsequent assessment of fertility and sperm quality in adulthood [46]. Long-term, follow up studies looking at testis biopsies from survivors of childhood cancers are extremely rare: one study has shown a reduction of germ cells in a 5-year follow up after treatment that included alkylating agents and doxorubicin [47], while another found no evidence of damage in a 10-year follow up after treatment with the alkylating agent cyclophosphamide [48]. In order to assess whether the acute SSC damage caused by cisplatin or doxorubicin would have repercussions on later phases of spermatogenesis, we evaluated key stages of germ cell development. The density of spermatocytes dropped relative to controls one week after cisplatin exposure, temporarily recovered one month after exposure, to drop again two months later. A slightly different scenario occurred for doxorubicin treated tissues, with a reduction in spermatocyte density relative to controls at the highest concentration only in the first week and the two highest concentrations one month after drug exposure, but with no significant difference found at the end of the two months of culture (although a non-statistically significant dose-response trend was present). Moreover, after drug treatment, a lower proportion of testis fragments contained round or elongated spermatids, at four and eight weeks respectively. These findings are in line with the reduced probability of siring a pregnancy observed in male cancer survivors who received higher doses of cisplatin during childhood [4]. Furthermore, it is important to bear in mind that patients will routinely receive multiple cycles of chemotherapy treatment, therefore any recovery of the germ cell population after a single acute gonadotoxic insult will then be exposed to further doses of chemotherapy agents.

This study has examined the effect of changes to the experimental method for the long-term culture of prepubertal mouse testis, improving its use for reproductive toxicological assessment. Spermatogenesis is an intricate biological process, relying on the interdependent activity of different somatic cell types, all contributing to the development of male germ cells: SSCs balance self-renewal and differentiation, culminating with the production of sperm from puberty in humans. For more than a century, attempts have been made to reproduce this complex cascade of events *in vitro* (history summarised in [49]), with improvements to a culture system that supports this process still the focus of investigations, both to help understand the process and also as a potential fertility treatment. The organ culture used here is slightly modified from the ground-breaking system developed by Sato and colleagues [25], capable of activating and maintaining spermatogenesis in neonatal mouse testis, while preserving cellular interactions and the microenvironment through retention of its three-dimensional architecture. Here, an air-liquid interphase has been used, with testicular fragments floating on polycarbonate membrane; this has the advantage of increasing the stability of fragment position, along with a reduction in handling-time for the operator. The other main change here is to the reduced size of tissue fragments used at the start of the culture, which has improved testicular integrity and reduced the occurrence of necrosis in the centre of the tissue fragments. Maintaining testicular tissue in culture for an extended period of time can lead to a significant necrotic central area [25,50], inevitably reducing the value of the culture system as a tool for toxicological screening. Here, the small average size of the fragments has likely increased nutrient perfusion to the tissue centre. The prolonged culture period has, however, increased heterogeneity of tubule morphology, with several expanded tubules likely the result of abnormal liquid accumulation due to altered trans-epithelial transport of luminal fluids. That in turn can negatively impact on germ cell development, increasing the occurrence of tubules with reduced germ cell population or even the appearance of Sertoli-cell-only tubules. Despite such discrepancies compared to *in vivo* tissue, others have successfully used this method to assess reproductive toxicity [51,52], and the changes outlined here further improve the usefulness of this method as toxicological tool. Future studies will be required to identify key medium components and strategies in order to continue to optimise culture conditions [53,54]. Comparative *in vivo* studies, taking into account the pharmacokinetics of chemotherapy agents, would be useful to validate the *in vitro* system.

In conclusion, these data show that treatment with either cisplatin or doxorubicin results in acute, direct damage to SSCs. Crucially, despite a recovery in SSC number in the longer term, the negative consequences of exposure to higher concentrations of cisplatin were still marked around the period of the second wave of spermatogenesis, with reduced number of total germ cells, both pre- and post-meiosis. The study has also made a key step forward in the improvement of an organ culture system, so that it supports full spermatogenesis from immature mouse testis more reliably and consistently, improving its usefulness for toxicological investigations on the developing testicular tissue.

Author contributions

FL participated in the design of the study, led experiments, prepared figures and wrote the manuscript. PT participated in the experiments and helped draft the manuscript. SKA and RAA helped design the study and draft the manuscript. RTM and NS conceived and coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

Funding

Work was supported by Children with Cancer UK grant 15-198, United Kingdom. RTM and RAA's work was undertaken in the Medical Research Council (MRC) Centre for Reproductive Health funded by MRC Centre Grant MR/N022556/1, United Kingdom.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Crispin Jordan for advices on statistical analysis, and to Melanie McMillan and Laura Johnstone at the Shared University Research Facilities (SuRF) at the University of Edinburgh for their

support with the PAS staining. We also thank Vivian Allison, Louise Dunn and Mike Molinek, staff at the Centre for Discovery Brain Sciences for their assistance with logistics for performing the experiments.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.12.023.

References

[1] M. Delessard, J. Saulnier, A. Rives, L. Dumont, C. Rondanino, N. Rives, Exposure to chemotherapy during childhood or adulthood and consequences on spermatogenesis and male fertility, Int. J. Mol. Sci. 21 (2020).
[2] G. Gatta, R. Capocaccia, M.P. Coleman, L.A. Ries, F. Berrino, Childhood cancer survival in Europe and the United States, Cancer 95 (2002) 1767–1772.
[3] A.B. Mariotto, J.H. Rowland, K.R. Yabroff, S. Scoppa, M. Hachey, L. Ries, E. J. Feuer, Long-term survivors of childhood cancers in the United States, Cancer Epidemiol. Biomarkers Prev. 18 (2009) 1033–1040.
[4] E.J. Chow, K.L. Stratton, W.M. Leisenring, K.C. Oeffinger, C.A. Sklar, S. S. Donaldson, J.P. Ginsberg, L.B. Kenney, J.M. Levine, L.L. Robison, M. Shnorhavorian, M. Stovall, G.T. Armstrong, D.M. Green, Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor Study cohort, Lancet Oncol. 17 (2016) 567–576.
[5] C.A. Knapp, G.P. Quinn, D. Murphy, Assessing the reproductive concerns of children and adolescents with cancer: challenges and potential solutions, J. Adolesc. Young Adult Oncol. 1 (2011) 31–35.
[6] N.E. Langeveld, M.A. Grootenhuis, P.A. Voute, R.J. de Haan, C. van den Bos, Quality of life, self-esteem and worries in young adult survivors of childhood cancer, Psychooncology 13 (2004) 867–881.
[7] C.M. Allen, F. Lopes, R.T. Mitchell, N. Spears, How does chemotherapy treatment damage the prepubertal testis? Reproduction (Cambridge, England) 156 (2018) 209–233.
[8] E. Goossens, K. Jahnukainen, R.T. Mitchell, A. van Pelt, G. Pennings, N. Rives, J. Poels, C. Wyns, S. Lane, K.A. Rodriguez-Wallberg, A. Rives, H. Valli-Pulaski, S. Steimer, S. Kliesch, A. Braye, M.M. Andres, J. Medrano, L. Ramos, S. G. Kristensen, C.Y. Andersen, R. Bjarnason, K.E. Orwig, N. Neuhaus, J. B. Stukenborg, Fertility Preservation in Boys: Recent Developments and New Insights (†), Human reproduction open 2020, 2020 hoaa016.
[9] E.H. Aksu, F.M. Kandemir, M. Ozkaraca, A.D. Omur, S. Kucukler, S. Comakli, Rutin ameliorates cisplatin-induced reproductive damage via suppression of oxidative stress and apoptosis in adult male rats, Andrologia 49 (2017).
[10] A. Carmely, D. Meirow, A. Peretz, M. Albeck, B. Bartoov, B. Sredni, Protective effect of the immunomodulator AS101 against cyclophosphamide-induced testicular damage in mice, Hum. Reprod. (Oxf., Engl.) 24 (2009) 1322–1329.
[11] S. Saral, E. Ozcelik, A. Cetin, O. Saral, N. Basak, M. Aydin, O. Ciftci, Protective role of Diospyros lotus on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats, Andrologia 48 (2016) 308–317.
[12] V. Vendramini, E. Sasso-Cerri, S.M. Miraglia, Amifostine reduces the seminiferous epithelium damage in doxorubicin-treated prepubertal rats without improving the fertility status, Reprod. Biol. Endocrinol. 8 (2010) 3.
[13] H.M. Picton, C. Wyns, R.A. Anderson, E. Goossens, K. Jahnukainen, S. Kliesch, R. T. Mitchell, G. Pennings, N. Rives, H. Tournaye, A.M. van Pelt, U. Eichenlaub-Ritter, S. Schlatt, A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys, Hum. Reprod. (Oxf., Engl.) 30 (2015) 2463–2475.
[14] M. Poganitsch-Korhonen, I. Masliukaite, M. Nurmio, P. Lähteenmäki, M. van Wely, A.M.M. van Pelt, K. Jahnukainen, J.B. Stukenborg, Decreased spermatogonial quantity in prepubertal boys with leukaemia treated with alkylating agents, Leukemia 31 (2017) 1460–1463.
[15] M. Nurmio, J. Toppari, J. Kallio, M. Hou, O. Söder, K. Jahnukainen, Functional in vitro model to examine cancer therapy cytotoxicity in maturing rat testis, Reprod. Toxicol. (Elmsford, N.Y.) 27 (2009) 28–34.
[16] W.H. Wallace, S.M. Shalet, E.C. Crowne, P.H. Morris-Jones, H.R. Gattamaneni, D. A. Price, Gonadal dysfunction due to cis-platinum, Med. Pediatr. Oncol. 17 (1989) 409–413.
[17] G.J. Bubley, J. Xu, N. Kupiec, D. Sanders, F. Foss, M. O'Brien, Y. Emi, B.A. Teicher, S.R. Patierno, Effect of DNA conformation on cisplatin adduct formation, Biochem. Pharmacol. 51 (1996) 717–721.
[18] J.N. van den Anker, How to improve the safe and effective use of doxorubicin in children with cancer, Clin. Pharmacokinet. 54 (2015) 1091–1093.
[19] A.A. Hussein, N.D. Tran, J.F. Smith, Fertility preservation for boys and adolescents facing sterilizing medical therapy, Transl. Androl. Urol. 3 (2014) 382–390.
[20] E. Smart, F. Lopes, S. Rice, B. Nagy, R.A. Anderson, R.T. Mitchell, N. Spears, Chemotherapy drugs cyclophosphamide, cisplatin and doxorubicin induce germ cell loss in an in vitro model of the prepubertal testis, Sci. Rep. 8 (2018) 1773.
[21] A.L. Drumond, C.C. Weng, G. Wang, H. Chiarini-Garcia, L. Eras-Garcia, M. L. Meistrich, Effects of multiple doses of cyclophosphamide on mouse testes: accessing the germ cells lost, and the functional damage of stem cells, Reprod. Toxicol. (Elmsford, N.Y.) 32 (2011) 395–406.
[22] B.P. Hermann, M. Sukhwani, C.C. Lin, Y. Sheng, J. Tomko, M. Rodriguez, J. J. Shuttleworth, D. McFarland, R.M. Hobbs, P.P. Pandolfi, G.P. Schatten, K. E. Orwig, Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques, Stem Cells (Dayton, Ohio) 25 (2007) 2330–2338.
[23] J.B. Stukenborg, J.P. Alves-Lopes, M. Kurek, H. Albalushi, A. Reda, V. Keros, V. Töhönen, R. Bjarnason, P. Romerius, M. Sundin, U. Norén Nyström, C. Langenskiöld, H. Vogt, L. Henningsohn, R.T. Mitchell, O. Söder, C. Petersen, K. Jahnukainen, Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy, Hum. Reprod. (Oxf., Engl.) 33 (2018) 1677–1683.
[24] M.L. Meistrich, Male gonadal toxicity, Pediatr. Blood Cancer 53 (2009) 261–266.
[25] T. Sato, K. Katagiri, A. Gohbara, K. Inoue, N. Ogonuki, A. Ogura, Y. Kubota, T. Ogawa, In vitro production of functional sperm in cultured neonatal mouse testes, Nature 471 (2011) 504–507.
[26] F. Lopes, R. Smith, S. Nash, R.T. Mitchell, N. Spears, Irinotecan metabolite SN38 results in germ cell loss in the testis but not in the ovary of prepubertal mice, Mol. Hum. Reprod. 22 (2016) 745–755.
[27] E.F. Oakberg, A new concept of spermatogonial stem-cell renewal in the mouse and its relationship to genetic effects, Mutat. Res. 11 (1971) 1–7.
[28] A.R. Bellvé, J.C. Cavicchia, C.F. Millette, D.A. O'Brien, Y.M. Bhatnagar, M. Dym, Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization, J. Cell Biol. 74 (1977) 68–85.
[29] W.R. Crom, W.E. Evans, C.B. Pratt, N. Senzer, M. Denison, A.A. Green, F.A. Hayes, G.C. Yee, Cisplatin disposition in children and adolescents with cancer, Cancer Chemother. Pharmacol. 6 (1981) 95–99.
[30] C. Dominici, F. Petrucci, S. Caroli, A. Alimonti, A. Clerico, M.A. Castello, A pharmacokinetic study of high-dose continuous infusion cisplatin in children with solid tumors, J. Clin. Oncol. 7 (1989) 100–107.
[31] B. Erdlenbruch, M. Nier, W. Kern, W. Hiddemann, A. Pekrun, M. Lakomek, Pharmacokinetics of cisplatin and relation to nephrotoxicity in paediatric patients, Eur. J. Clin. Pharmacol. 57 (2001) 393–402.
[32] B. Peng, M.W. English, A.V. Boddy, L. Price, R. Wyllie, A.D. Pearson, M.J. Tilby, D. R. Newell, Cisplatin pharmacokinetics in children with cancer, Eur. J. Cancer (Oxf., Engl.: 1990) 33 (1997) 1823–1828.
[33] B.M. Frost, S. Eksborg, O. Bjork, J. Abrahamsson, M. Behrendtz, A. Castor, E. Forestier, G. Lonnerholm, Pharmacokinetics of doxorubicin in children with acute lymphoblastic leukemia: multi-institutional collaborative study, Med. Pediatr. Oncol. 38 (2002) 329–337.
[34] S. Voller, J. Boos, M. Krischke, G. Wurthwein, N.E. Kontny, A.V. Boddy, G. Hempel, Age-dependent pharmacokinetics of doxorubicin in children with cancer, Clin. Pharmacokinet. 54 (2015) 1139–1149.
[35] R.P. Vergouwen, S.G. Jacobs, R. Huiskamp, J.A. Davids, D.G. de Rooij, Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice, J. Reprod. Fertil. 93 (1991) 233–243.
[36] C.M. Allen, F. Lopes, R.T. Mitchell, N. Spears, Comparative gonadotoxicity of the chemotherapy drugs cisplatin and carboplatin on prepubertal mouse gonads, Mol. Hum. Reprod. 26 (2020) 129–140.
[37] R. Shabani, K. Ashtari, B. Behnam, F. Izadyar, H. Asgari, M. Asghari Jafarabadi, M. Ashjari, E. Asadi, M. Koruji, In vitro toxicity assay of cisplatin on mouse acute lymphoblastic leukaemia and spermatogonial stem cells, Andrologia 48 (2016) 584–594.
[38] S. Dasari, P.B. Tchounwou, Cisplatin in cancer therapy: molecular mechanisms of action, Eur. J. Pharmacol. 740 (2014) 364–378.
[39] C.F. Thorn, C. Oshiro, S. Marsh, T. Hernandez-Boussard, H. McLeod, T.E. Klein, R. B. Altman, Doxorubicin pathways: pharmacodynamics and adverse effects, Pharmacogenet. Genomics 21 (2011) 440–446.
[40] M. Hou, D. Chrysis, M. Nurmio, M. Parvinen, S. Eksborg, O. Söder, K. Jahnukainen, Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity, Cancer Res. 65 (2005) 9999–10005.
[41] L.C. Lirdi, T. Stumpp, E. Sasso-Cerri, S.M. Miraglia, Amifostine protective effect on cisplatin-treated rat testis, Anatomical Rec. (Hoboken, N.J. : 2007) 291 (2008) 797–808.
[42] O. Brilhante, F.K. Okada, E. Sasso-Cerri, T. Stumpp, S.M. Miraglia, Late morfofunctional alterations of the Sertoli cell caused by doxorubicin administered to prepubertal rats, Reprod. Biol. Endocrinol. 10 (2012) 79.
[43] F.K. Okada, T. Stumpp, S.M. Miraglia, Carnitine diminishes etoposide toxic action on spermatogonial self-renewal and sperm production in adult rats treated in the prepubertal phase, J. Histochem. Cytochem. 68 (2020) 327–342.
[44] J.G. Harman, J.H. Richburg, Cisplatin-induced alterations in the functional spermatogonial stem cell pool and niche in C57/BL/6J mice following a clinically relevant multi-cycle exposure, Toxicol. Lett. 227 (2014) 99–112.
[45] M.L. Meistrich, Relationship between spermatogonial stem cell survival and testis function after cytotoxic therapy, Br. J. Cancer 7 (Supplement) (1986) 89–101.
[46] R.E. Cabral, F.K. Okada, T. Stumpp, V. Vendramini, S.M. Miraglia, Carnitine partially protects the rat testis against the late damage produced by doxorubicin administered during pre-puberty, Andrology 2 (2014) 931–942.
[47] J. Müller, H. Hertz, N.E. Skakkebaek, Development of the seminiferous epithelium during and after treatment for acute lymphoblastic leukemia in childhood, Horm. Res. 30 (1988) 115–120.
[48] A.J. Pennisi, C.M. Grushkin, E. Lieberman, Gonadal function in children with nephrosis treated with cyclophosphamide, Am. J. Dis. Child. 129 (1975) 315–318 (1960).
[49] K. Reuter, S. Schlatt, J. Ehmcke, J. Wistuba, Fact or fiction: in vitro spermatogenesis, Spermatogenesis 2 (2012) 245–252.
[50] R.E. Chapin, T. Winton, W. Nowland, N. Danis, S. Kumpf, K. Johnson, A. Coburn, J. B. Stukenborg, Lost in translation: the search for an in vitro screen for

spermatogenic toxicity, Birth Defects Res. B Dev. Reprod. Toxicol. 107 (2016) 225–242.
[51] N. Nakamura, D.T. Sloper, P.L. Del Valle, Evaluation of an in vitro mouse testis organ culture system for assessing male reproductive toxicity, Birth Defects Res. 111 (2019) 70–77.
[52] H.J. Park, W.Y. Lee, M. Zhang, K.H. Hong, C. Park, J.H. Kim, H. Song, Evaluation of resmethrin toxicity to neonatal testes in organ culture, Toxicol. Sci. 173 (2020) 53–64.
[53] J. Isoler-Alcaraz, D. Fernández-Pérez, E. Larriba, J. Del Mazo, Cellular and molecular characterization of gametogenic progression in ex vivo cultured prepuberal mouse testes, Reprod. Biol. Endocrinol. 15 (2017) 85.
[54] H. Sanjo, M. Komeya, T. Sato, T. Abe, K. Katagiri, H. Yamanaka, Y. Ino, N. Arakawa, H. Hirano, T. Yao, Y. Asayama, A. Matsuhisa, M. Yao, T. Ogawa, In vitro mouse spermatogenesis with an organ culture method in chemically defined medium, PLoS One 13 (2018) e0192884.