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Germ Cell Responses to Doxorubicin Exposure *in Vitro*

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Highlights

- Purified cells were treated with different concentrations of the genotoxic agent Doxorubicin (Dox) and assessed for DNA damage and apoptosis.
- Exposure of the purified germ cells to Dox yielded significant increases in DNA damage and apoptosis.
- Dox disrupts spermatogenesis by causing DNA damage and apoptosis in spermatogonia, spermatocytes and spermatids.
- The effects of Dox were cell type- and exposure-dependent with the strongest responses at the highest concentration in spermatogonia and a lack of response in spermatids at the lowest concentration.

Abstract

Anthracyclines such as doxorubicin (Dox), widely used to treat various types of tumours, may result in induced testicular toxicity and oxidative stress. The present investigation was designed to determine whether exposure of isolated and purified mouse germ cells to Dox induces DNA damage in the form of strand breaks (presumably) resulting in apoptosis and to investigate the relative sensitivity of specific cell types. DNA damage was assessed using the Comet assay and the presence of apoptosis was determined by TUNEL assay. Isolated mouse germ cells were treated with different concentrations (0.05, 0.5 and 1 mM, respectively) of Dox, and fixed 1 h after treatment. The incidences of both DNA damage shown by single cell gel-electrophoresis and of apoptosis increased significantly in each specific cell type in a concentration-dependent manner. The DNA damage and apoptosis incidences gradually increased with concentration from 0.05 to 1 mM with Dox. Our results indicate that apoptosis plays a vital role in the induction of germ cell phase-specific toxicity caused by Dox with pre-meiotically and meiotically dividing spermatogonia and spermatocytes respectively as highly susceptible target cells.

Keywords: DNA damage; apoptosis; doxorubicin; Comet assay; TUNEL assay; male germ cells.

1. Introduction:

A frequently used chemotherapeutic drug is the extremely effective anthracycline doxorubicin (Dox) also known as adriamycin (Badkoobeh et al., 2013). It is the antineoplastic drug of choice in the treatment of various types of tumours such as, childhood leukemia and testicular cancer, though one of its adverse effects is male infertility (Shamberger et al., 1981, Imahie et al., 1995, Prahalathan et al., 2005, Vendramini et al., 2010).

Dox is well known to be a mutagen in both somatic cells (Smith, 2003, Pulte et al., 2008) and in early spermatogenic cells (Sjoblom et al., 1998, Zanetti et al., 2007).

Even a low dose of Dox (1.0 mg/kg) damaged mouse spermatogonia, though that dose was ineffective on primary spermatocytes (Lu & Meistrich, 1979). One of the responses to DNA damage induced by Dox is apoptosis and the induction of apoptosis in the adult testis is often one of the earliest signs of genotoxic damage (Jahnukainen et al., 2000). In the neonatal rodent testis, however, spontaneous apoptosis is extremely important for the maintenance of appropriate germ-cell number relative to Sertoli cell number (Rodriguez *et al*., 1997). In addition, various endogenous, normal physiological processes such as insulin signalling (Dias et al., 2013) can influence levels of testicular cell apoptosis.

Dox exerts an effect on spermatogonial cells mainly because of their high division rate and slow cell cycle (Brilhante et al., 2012). Recent study has shown that the higher mitotic turnover is required for spermatogonia to generate the same number of differentiated germ cells as species with a lower turnover rate but a higher number of differentiating spermatogonial generations (Ehmcke et al., 2006, Krieger & Simons, 2015). This higher mitotic turnover could increase the risk for germ cell mutations and vulnerability to cytotoxic events (Waheeb & Hofmann, 2011).

A wide range of clinical and experimental studies have demonstrated the testicular toxicity caused by Dox (Damani et al., 2002). It has been found that even a low dose of Dox (1 mg/kgb.w.) given to adult mice is able to target germ cells, mainly spermatogonia, leading to seminiferous epithelium depletion (Jahnukainen *et al*., 2000). The intercalation of Dox into germ cell DNA during division is considered to be the principal cause of cellular death in the seminiferous epithelium and influences the number of germ cells located in the vicinity of the basement membrane of the testis (Vendramini *et al*., 2010). Blood-testis barrier injury from Dox exposure, mediated by the generation of free radicals, has also been reported (Jahnukainen *et al*., 2000). It also exerts its effects via a mechanism that includes intercalation with DNA and consequent inhibition of topoisomerase II (Topo II) activity, which results in replication-dependent, site-selective double-strand breaks in DNA (Myers CE, 1990, Quiles et al., 2002) because anthracyclines inhibit the re-ligation of these breaks (Zunino & Capranico, 1990). Dox has also been shown to interfere transcription and the stability of chromosomes, by affecting DNA methyl transferase 1 activity, inducing apoptosis (Yokochi & Robertson, 2004), which also makes it potent in the developing germ cell line, leading to male infertility (Dacunha et al., 1983, Meistrich,

2013a). This in turn leads to up-regulation of p53, which prevents DNA replication in the presence of DNA damage and can thus lead to apoptosis (Bunz et al., 1998). Dox can also reduce the viability of cancer cells through RNA damage (Fimognari et al., 2008).

The generation of free radicals by Dox arises from its ability to bind iron and form complexes with DNA, thus resulting in DNA damage (Eliot et al., 1984, Ravi & Das, 2004) (Injac & Strukelj, 2008). This free radical generation from Dox causes genotoxicity in normal cells (Quiles et al., 2002) and in different types of cancer cells (Gouaze et al., 2001b). The induction of apoptosis in the adult testis is often one of the earliest signs of genotoxic damage (Jahnukainen et al., 2000). In the neonatal rodent testis, however, spontaneous apoptosis is extremely important for the maintenance of appropriate germ-cell number relative to Sertoli cell number (Rodriguez et al., 1997). In addition, various endogenous, normal physiological processes such as insulin signalling (Dias et al., 2013) can influence levels of testicular cell apoptosis. Exposure to Dox induces intracellular oxidative stress that can be ameliorated via the overexpression of antioxidant enzymes that prevent apoptosis in tumour cells (Suresh et al., 2003). Blocking the activity of endogenous antioxidants, such as glutathione peroxidase-1 produced by tumour cells, or depletion of glutathione pools also enhances the sensitivity of tumour cells to Dox (Gouaze et al., 2001a, Poirson-Bichat et al., 2000). It is therefore appropriate to investigate the adverse effects of Dox on susceptible normal cells such as spermatogenic cells, which are likely to be vulnerable to damage in a similar way as the tumour cells targeted during treatment.

2. Materials and Methods:

2.1.1. Animals

Male NMRI mice (National Medical Research Institute) 12 wk of age were obtained from the Institute of Cancer Therapeutics, University of Bradford, UK where they were maintained under standard conditions. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.1.2. Chemicals

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich, Poole, UK).

2.2. Preparation of cells and culture

The method for fractionation of mouse testicular germ cells was as described previously (Habas et al., 2014). Briefly, testes were collected from four male adult (10–12 week-old) NMRI mice, decapsulated, and the seminiferous tubules placed into ice cold Dulbecco's Modified Eagle's medium (DMEM), dispersed by gentle pipetting, minced and resuspended in fresh DMEM containing collagenase (5mg/ml) and DNAse (1µg/ml), then incubated at 32°C for 20 min. The cells were left to stand for 5 min before being filtered through an 80 µm nylon mesh (Tetco Inc., Briarcliff Manor, NY), centrifuged at 600 × g for 10 min and bottom-loaded into the separation chamber of a Staput apparatus in a volume of 10ml. A 2-4% w/v concentration gradient of BSA was then generated below the cells, which were allowed to sediment for a standard period of 2.5h before 31 separate 12ml fractions were collected at 60s intervals. The cells in each fraction were examined under a phase contrast microscope, and consecutive fractions containing cells of similar size and morphology spun down by low-speed centrifugation and resuspended in DMEM. The identity and purity of all cell preparations used in the experiments was confirmed by Reverse Transcription PCR assay (RT-PCR) and quantitative Reverse-Transcription PCR assay (RT-qPCR) as described below in sections 2.3 and 2.6 respectively. The viabilities of the freshly isolated spermatogonia, spermatocytes and spermatids were routinely >95%, as evidenced by trypan blue exclusion (Phillips, 1973) of these cells. The germ cells were cultured overnight at 37°C. The following day, viability was re-checked and the cells treated with mutagen as required (section 2.4). Viabilities were checked again and were found to be routinely >89% for cells that had been exposed to Dox. They were then used immediately for qPCR, Comet assay or TUNEL assay.

2.3. Confirmation of identity of purified, mouse spermatogonia, spermatocytes and spermatids by RT-PCR and RT-qPCR.

The identity and purity of all cell preparations used in the experiments was confirmed by RT-PCR for the presence or absence of spermatogonial-, spermatocyte- and spermatid-specific mRNA. Thus, total RNA was extracted from the freshly isolated mouse spermatogonia, spermatocytes, spermatids, and mouse testis tissues, using

TRIzol reagent (Invitrogen Carlsbad, CA), and total RNA quantity and quality was checked using $OD_{260/280}$ measurements. Reverse transcription (RT) was performed as described below in section 2.5.

Portions of genes specifically expressed in each of the main categories of male germ cells were amplified from cDNA produced as described above by PCR using primers for glial cell line derived neurotrophic factor receptor (GDNFR) (spermatogonia), synaptonemal complex protein 3 (SCP3) (spermatocytes), Transition protein-1 TP1 (spermatids) and β-actin for RT-PCR. (RT-qPCR was also performed for these genes: see section 2.6 for details.) The PCR reactions started with a single step of 94°C for 2 min, which was followed by the following cycle pattern: denaturation at 94°C for 30 sec, annealing at 58–61°C (depending on the primer pair) for 30 sec, and elongation at 72°C for 30 sec. After 30 cycles, the samples were incubated for an additional 5 min at 72°C. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide. Images were recorded and band intensities analysed using a digital gel documentation system (UVItec, Cambridge, UK).

2.4. Treatment with Dox

Freshly isolated germ-cell suspensions (1.5-2.5x10^5 cell/ml) were mixed with fresh RPMI medium (total volume 1000 µl). 100 µl of spermatogonia, spermatocytes and spermatids were then added to each treatment tube (100 µl spermatogonia, spermatocytes and spermatids, 890 µl RPMI medium, plus 10 µl of chemical or 900 µl for the negative control). Cells were treated with different concentrations (0, 0.05, 0.5, and 1mM) of Dox for 1 h at 37°C. Treated and untreated germ cells were then immediately subjected to RNA extraction or used in Comet or TUNEL assays.

2.5. Isolation of total RNA and cDNA synthesis

To remove any genomic DNA, the RNA was treated with DNase I according to the manufacturer's instructions. Random-hexamer primed, reverse transcription reactions were performed on 400 ng of total RNA in a 20 µl setup using ImProm-II™ Reverse Transcription System reaction following the manufacturer's instructions (Promega Co., WI, USA). The synthesised cDNA samples were diluted 1:10 in nuclease-free water and stored at −20°C.

2.6. Quantitative Reverse-Transcription PCR assay (RT-qPCR)

Reactions were carried out using the StepOnePlus™ real-time PCR instrument (Applied Biosystems). qPCR was used to quantify the mRNA expression of GDNFR, SCP3, TP1, p53 and Bcl2 in spermatogonia, spermatocytes and spermatids. Reactions were prepared in triplicates of 20 µl reaction mixtures in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). Each reaction well contained 2 µl template DNA, 2 µl 10x SYBR® Green PCR Master Mix (Applied Biosystems), and 12.5 pmol each of forward and reverse primers. Real-time qPCR was conducted with the following cycling conditions: 50°C for 2 min, 95°C for 20 s, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s each. The data obtained from each reaction was analysed by StepOne™ Software v2.2.2. Relative quantification representing the change in gene expression between experimental groups was calculated by comparative CT method. The data were analysed by calculating the relative quantification (RQ) using the equation: RQ = 2-ΔCT x100, where ΔCT = CT of target gene minus the CT of an endogenous housekeeping gene. Evaluation of 2-ΔCT indicates the fold change in gene expression, normalized to the internal control (β-actin), which enables valid comparison of differently treated cells.

2.7. Comet assay

Purified germ cells (spermatogonia, spermatocytes and spermatids) were assayed for DNA damage using alkaline single-cell electrophoresis (Comet) assay using the method described by Anderson et al., (1997) with slight modifications. Briefly, after treatment, isolated germ cells were centrifuged and the supernatant discarded bar small amounts retained with the cell pellet. Next, 100 µl of 1% low melting-point agarose (LMP) (Invitrogen, Paisley, UK: 15517-022) was added to the cell pellet to create a cell suspension. The cell suspension was transferred to slides pre-coated with 1% normal melting point (NMP) agarose. The slides were placed on an ice block for 5 min, after which 100 µl of 0.5% LMP was added on top and slides were placed on ice for 5 min. The slides then were submersed in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl pH 10.0 containing 1% Triton X-100 and 40 mM dithiothreitol) for 1 h at room temperature and protected from light to prevent damaging due to light. Following this initial lysis period, proteinase K (Zini & Sigman)

was added to the lysis solution (final concentration 10µg/ml) and additional lysis was performed at 37°C for 2.5 h (Hughes et al., 1997). Following lysis, slides were placed in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 30 min then subjected to electrophoresis at 20 V (approximately 300 mA) for 30 min at 4°C. After electrophoresis, slides were neutralised with 3 x 5 min rinses in Tris buffer. Each slide was stained with 50 µl of 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip.

Visual and computerized image analyses of DNA damage were carried out in accordance with the protocols of (Anderson & Plewa, 1998). Samples were run in triplicate, and 100 cells were randomly analysed per slide at 200X magnification with an Olympus fluorescent microscope (Andor Technology Ltd, Belfast, UK). Equipped with a BP546/10 excitation filter and a 590 nm barrier filter. Slides were analysed by a computerized image analysis system (Comet 6.0; Andor Technology, formerly Kinetic Imaging) Belfast, UK. Comet tail length is the maximum distance the damaged DNA migrates from the centre of the cell nucleus, and the tail moment is a product of the tail length and the percentage of tail DNA, which gives a more integrated measurement of overall DNA damage in the isolated germ cells.

2.8. TUNEL assay

Nuclear fragmentation consistent with apoptosis was determined by the TUNEL assay as previously described in Habas *et al.* (2014) using the Terminal Deoxynucleotidyl Transferase (TdT) Kit. Isolated germ cells were cultured and then incubated with TUNEL reaction mixture (30 mM Tris pH 7.4; 140 mM sodium cacodylate; 1 mM cobalt chloride; 5 µM biotin-16-deoxyuridine triphosphate; 0.3U/µl terminal deoxynucleotidyl transferase (TDT) for 60 min (humidity chamber, 37°C). Cells were scored as TUNEL positive if they appeared as condensed cells with dark-brown stained nuclei following detection of the label with Extravidin peroxidase and visualisation with diamino benzidine. In all assays the apoptotic cells are calculated as the percentage of TUNEL positive cells and represent at least 100 cells counted and are expressed as the mean ± SEM.

2.9. Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments with three replicates per experimental group. Comparisons were made by one-way ANOVA followed by Bonferroni *post hoc* test; for all experiments, a *P* value of <0.05 was considered significant. All analyses were performed using SPSS for windows statistical package (version 18.0).

3. Results

3.1. Gene expression in isolated spermatogonia, spermatocytes and spermatids assessed by RT-PCR and RT-qPCR.

To assess the purities of freshly isolated cells, cell type-specific gene expression was used as previously (Habas et al., 2014, 2016) except that RT-PCR and RT-qPCR amplification of the specific genes was used rather than immunoblotting or immunocytochemistry. RT-PCR (Figure 1) demonstrated that GDNFR transcripts were present in the spermatogonial cells whereas SCP3 and TP1 were undetected. SCP3 transcripts were present in the spermatocytes, whereas GDNFR and TP1 were not. Likewise TP1 but not GDNFR or SCP3 was found to be expressed in the spermatids. The expression of these genes in mouse testis tissues served as positive controls. B-actin served as a loading control of total RNA (Figure 1). The results were also confirmed by RT-qPCR (Figure2), which additionally gives an indication of the purity of the cell preparations.

3.2. Quantification by RT-qPCR of gene expression in response to treatment

For the qPCR assay, different levels of expression of p53 (Figure 3) and Bcl-2 (Figure 4) mRNA in spermatogonia, spermatocytes and spermatids were determined after treatment with different concentrations of Dox. The samples were taken at 24 h following Dox treatment for both treated and untreated control cultures, and the expression levels of p53 and Bcl-2 were normalised against those of β-actin and compared with the equivalent control (0mM Dox) value.

As the Dox concentration increased from 0 to 1 mM, the mRNA level for p53 was found to be increased in spermatogonia, spermatocytes and spermatids while the mRNA levels of Bcl-2 decreased. There were statistically significant differences in the levels of both p53 and Bcl-2 at 0.05, 0.5 and 1 mM Dox in each cell types also different between three types of cells.

There was a statistically significant increase in the levels of p53 RNA after 24 h of treatment with 0.05 mM Dox in spermatogonia ($p<0.01$). Further significant increases were seen in spermatogonia treated with 0.5 mM or 1 mM Dox ($p<0.001$) as shown in Figure 3. In contrast, p53 was only significantly elevated in spermatocytes and spermatids treated with 0.5 mM ($p<0.01$ and $p<0.05$ respectively) or 1 mM Dox ($p<0.001$).

Twenty-four hours after treatment of spermatogonia with 0.05 mM Dox, there was a statistically significant decrease in the levels of Bcl-2 RNA ($p<0.05$). This was more marked by treatment with 0.5 mM and 1 mM Dox ($p<0.001$) as shown in Figure 4. As with p53 RNA levels, Bcl-2 was unchanged in spermatocytes and spermatids treated with 0.05mM Dox but it was decreased by 0.5 mM and 1 mM Dox ($p<0.01$ and $p<0.001$ respectively) in both cell types.

3.3. Quantification of DNA breaks by the Comet assay

Olive tail moment (OTM) and %tail DNA are shown in Figures 5 and 6. A significant increase from 0.68 in control to 2.5 (OTM) and 26.6 in control to 37.2 (%tail DNA) was observed in the OTM and %tail DNA of spermatogonia treated with 0.05 mM Dox. At 0.5 mM, OTM and %tail DNA damage also showed a significant increase to 4.9 and 46% respectively in spermatogonial cells compared with control ($p \leq 0.001$) while with 1 mM, DNA damage increased further to 7.4 for OTM and 59.35 % for tail DNA. In contrast, spermatocytes showed no significant increase in either Comet parameter at 0.05 mM Dox. They did however show significant increases in the OTM and %tail DNA at 0.5 mM Dox to 3.8 for OTM and 40.97 % for tail DNA ($p \leq 0.001$). The values increased further to 5.7 for OTM and 43.61 % for tail DNA at 1 mM ($p \leq 0.001$). Treatment of spermatids also showed no increase with 0.05 mM Dox and with 0.5 mM there was only a significant increase in the OTM ($p \leq 0.05$) but not in % tail DNA when compared with the corresponding controls. However, in spermatids treated with 1 mM Dox the DNA damage increased to 4.25 for OTM and 42.21 % for tail DNA (both $p \leq 0.01$).

3.4. Quantification of apoptosis by TUNEL assay

The result of Dox treatment on isolated germ cells of mouse testis was expressed as mean percentage of apoptotic cells per group ± SEM. The TUNEL assay revealed

that all cells types had undergone significant levels of apoptosis compared with the controls (Figure 7). After treatment for 1 h, a significant increase ($p \leq 0.01$) was observed in spermatogonial apoptosis from 6 % in the controls to 12 % when cells were treated with 0.05 mM Dox. This increased to 23 % when treated with 0.5 mM Dox ($p \leq 0.001$) and to 38 % when the cells were treated with 1 mM Dox. Following treatment with 0.05 mM Dox, spermatocytes showed no statistically significant increase in apoptosis but at 0.5 mM Dox, they showed an increase to 15 %, which was significant when compared with the corresponding controls ($p \leq 0.01$). A further increase to 34 % in cell apoptosis was observed when spermatocytes were treated with 1 mM Dox ($p \leq 0.001$). As with the spermatocytes, apoptosis of spermatids treated with 0.05 mM Dox showed no statistically significant increase but at 0.5 mM Dox, it was significantly increased to 13 % compared with the corresponding controls ($p \leq 0.05$) and to 24 % when cells were treated with 1 mM Dox ($p \leq 0.01$). A qualitative comparison of Figures 5 & 6 with Figure 7 shows that broadly the same pattern of differential responses was observed with apoptosis as with DNA damage.

4. Discussion

Infertility and subfertility are the most well-known long-term side effects to the chemotherapy drugs. Previous studies in mice, rat and humans has shown that chemotherapy can result in decreased sperm count and reproductive capacity (Lee & Shin, 2013). Improved survival cells with modern chemotherapy drugs have increased survival with modern chemotherapeutics has high the anticipation of fertility preservation in cancer survivors (Lee & Shin, 2013). Dox is one of common drugs used in the treatment of cancers, also alone or in combination with other chemotherapeutic drugs. This drug induce cancer cell apoptosis also DNA damage male germ cells (Jia et al., 2015). In the present study, we aimed to investigate the usefulness of *in vitro* germ cell culture in combination with the Comet and TUNEL assays, to determine DNA damage and apoptotic cell death induced by a genotoxic agent in male germ cells in mice. As the genotoxic effect of Dox on male germ cells is well recognized, it was suitable for use in this work and the results may also be useful for approaches to the prevention of unintended damage in the germline. The toxic action of Dox on male germ cells has been described (Vendramini et al., 2010). The citotoxity caused by Dox on the germ cell can be interrelated to its therapeutic activity; it interferes with molecules associated to the nuclear DNA and with enzymes

RNA and DNA polymerase, topoisomerases I and II which are active in the cell division process. Then, doxorubicin forms a complex with chromatin (Rabbani et al., 2005), blocking the G2 phase of the cell cycle (van Rosmalen et al., 1995), and provoking single and/or double strand DNA breaks (Ross and Bradley, 1981).

Examination of isolated, mouse germ cells treated with Dox in the present study indicated a significant reduction in the cytotoxicity and cells apoptosis. The toxic action of Dox on male germ cells has been described (Vendramini et al., 2010). The citotoxity caused by Dox on the germ cell can be interrelated to its therapeutic activity; it interferes with molecules related to the nuclear DNA and with enzymes (RNA and DNA polymerases, topoisomerases I and II) that are active in the cell division process (Brilhante et al., 2012). Consequently, forms a complex with chromatin. The evidence implies that one very important component of the activity of Dox is the result of these manifold interactions that lead to a chromatin unfolding and aggregation. This chromatin structural disruption is possible to interfere with the metabolic processes of DNA (replication and transcription) and it might play a critical role in the apoptosis undergone via the cells upon treated with Dox (Rabbani et al., 2005). Dox induced cell death by blocking the cell cycle at the G2 phases (van Rosmalen et al., 1995, Zhou et al., 2012). It also has ability to cause DNA single- and double-strand breaks (SSB, DSB) (Xu & Her, 2015).The incidence of morphological changes and nucleosomal degeneration of male germ cell nuclei demonstrate that they undergo apoptosis as revealed by in situ terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL assay) (Yeh et al., 2009). It has been shown that apoptosis is one of the mechanisms involved in cell destruction following chemotherapy (Hou et al., 2005). It is also well recognised that Dox induces apoptosis in early spermatogonia, which results in a decrease in the size of the pool of germ line stem cells (Hou et al., 2005). In addition, another study has shown that Dox causes damage to spermatogonia and spermatocytes (Vendramini, Sasso-Cerri and Miraglia, 2010). This potential infertility-causing complication renders protection of testicular tissue a critical issue whenever Dox is employed for anti-neoplastic chemotherapy. A previous study has shown that Dox impaired mouse testicular structure through inflicting oxidative stress and inducing cell apoptosis (Yeh et al., 2007). Understanding the biological mechanisms of germ cells injury caused by chemotherapy such as Dox is an important to developing new

reproductive system preservation strategies. However, one possible strategy to prevent Dox toxicity is inhibiting TOP II-mediated DNA cleavage to prevent accumulation of dsDNA breaks, while allowing time for the cell to metabolize and remove Dox (Kropp et al., 2015). Dox also can mediated cell cycle arrest either at G0/G1 or G2 check points and is thought to be mediated via the multifunctional transcription factor p53 (Lowe & Lin, 2000). p53 is can be activated by genotoxic stresses and regulates multiple cellular responses via transcriptional activation DNA repair and apoptosis (Vousden & Lu, 2002).

Chemotherapy for the treatment of cancer can have devastating effects on male reproduction (Meistrich, 2013b). Furthermore, fertility recovery is very hard to predict as it is dependent on the chemotherapeutic drugs and dosages used as well as individual susceptibility. Consequently, there is considerable interest in identifying agents or approaches that are able to preserve male fertility without interfering with the efficacy of the chemotherapeutic regimen (Rabaca et al., 2015). Furthermore, evaluation of possible gonadotoxicity and selection of the appropriate fertility preservation methods prior to the start of cancer therapy in individual patients is vital for limiting the anti-spermatogenic side-effects of treatment (Levine, 2011). Classification of the gonadotoxicity of current treatment regimens according to their various side effects on male reproductive function is also required, in order to aid improvements in intervention. Until more therapies and treatment regimens with minimal side-effects are developed, it is essential that all fertility preservation options are discussed with patients, before treatment is initiated, to assure continued fertility after recovery from the cancer treatment (Rabaca et al., 2015).

The present study adds to this knowledge by demonstrating that different cell types have different susceptibilities to Dox. Crucially, we have been able to demonstrate this without the need for time-consuming, resource-intensive, animal studies. In clinical practice, the Comet assay has been considered important tools to predict pregnancy outcome (Zini et al., 2008). However, the long-term effects of Dox association on human sperm DNA and its consequences to male fertility (Vendramini et al., 2012).

According to our results, Dox adversely damages testicular tissue and significantly can be reduced the sperm production through increasing DNA damage and inducing

apoptosis. Nevertheless, our results also show that the Comet assay is usable for the investigation of DNA damage induction in cultured cells and thus provides a useful system for genotoxicity evaluation of effects in different types of germ cells without relying solely on animal studies. As Dox based chemotherapy is still crucial for treatment of many types of cancers but drug induced infertility is often inevitable, our results highlight the fact that close attention must be paid to childhood cancer survivors who have plan a pregnancy if they were previously exposed to Dox, although our results are based on animal model.

5. Conclusion

In conclusion, the results presented in this study show that stage-specific separation of germ cells and their *in vitro* exposure to a range of Dox concentrations allows the detection of genetic damage and apoptosis in the different categories of male germ cells, using a range of end-points. This technique is rapid and does not need animal experimentation *in vivo*, in contrast to traditional techniques that employ histological morphology and fertility studies. Furthermore, because it only involves short term culture, it enables detection of the earliest signs of toxicity. This makes it useful in the detection of germline genotoxicity in drugs and other compounds of interest, and hence of value in safety evaluation procedures and in efforts to protect non-target cells against the general cytotoxicity of chemotherapeutic agents. Furthermore, by adding appropriate end-points, it is possible to gain mechanistic data that enable modes of action to be studied in the same investigation. Thus, our data confirm that Dox induces DNA damage and apoptosis (reflected by a decrease in Bcl2 expression) in male germ cells but also indicate that the actively proliferating germ cells, spermatogonia and, to a lesser degree, spermatocytes are more vulnerable than spermatids. In turn, this demonstrates a link between the induction of damage and cellular proliferation, which is reinforced by the finding of a matching, cell-type related, increase in p53 expression, especially at the lower concentrations of Dox.

7. Conflict of Interest Statement

The authors have no conflicts of interest with regard to the funding of this research.

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Figure1: RT-PCR analysis on Staput-purified testicular cells specific mRNA was detected in each isolated cell fraction by specific genes. RT-PCR displayed the genes of GDNFR, SCP3 and TP1 in spermatogonia, spermatocytes and spermatids respectively. B-actin served as a loading control of total RNA. The data shown were representatives from three independent experiments.

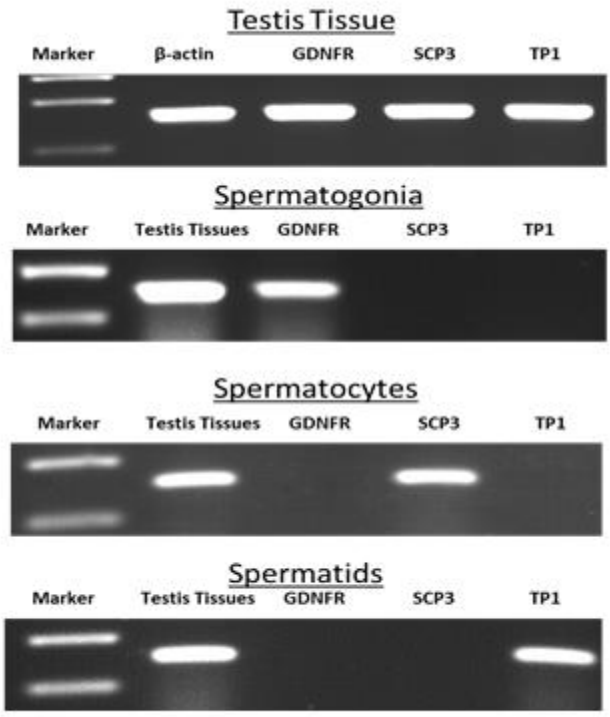

Figure 2: qPCR analysis on Staput-purified mouse testicular cells specific mRNA was detected in each isolated cell fraction by specific genes. QPCR displayed the genes of TP1, GDNFR and SCP3 in spermatogonia, spermatocytes and spermatids isolated from mouse testis tissues. The expression of these genes in mouse testis tissues served as positive controls. B-actin served as a loading control of total RNA. The data shown were representatives from three independent experiments.

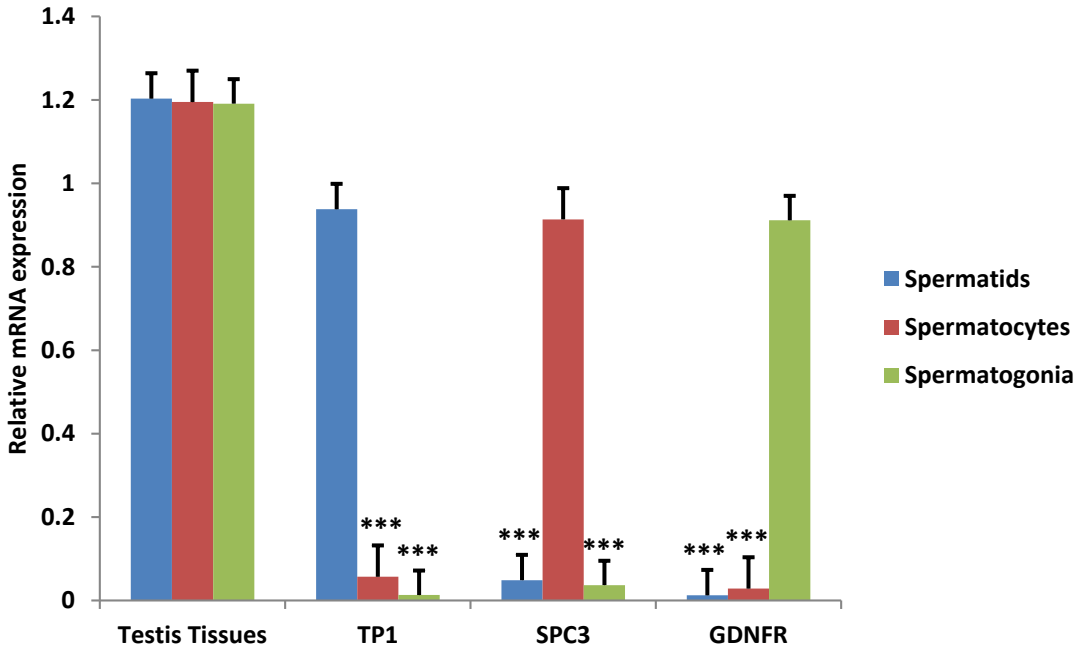

Figure 3: Concentration-dependent effects of Dox on p53 mRNA expression levels in spermatogonia, spermatocytes and spermatids, treated with different concentrations of Dox (0, 0.05, 0.5 and 1 mM). mRNA expression levels were determined by qPCR. β-actin mRNA was used as an internal control. The data shown are representative of three independent experiments. The significant differences from control are indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

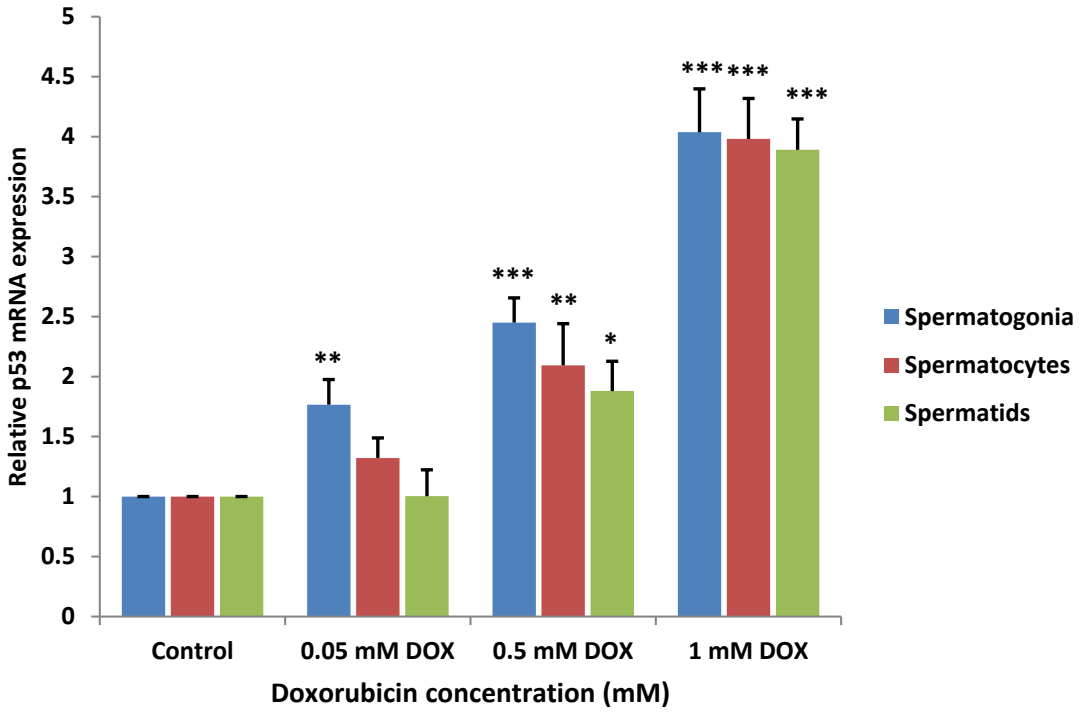

Figure 4: Concentration-dependent effects of Dox on Bcl-2 mRNA expression levels in spermatogonia, spermatocytes and spermatids, treated with different concentrations of Dox (0, 0.05, 0.5 and 1 mM). mRNA expression levels were determined by qPCR. β-actin mRNA was used as an internal control. The data shown are representative of three independent experiments. The significant differences from control are indicated by *P < 0.05, **P < 0.01, ***P < 0.001.

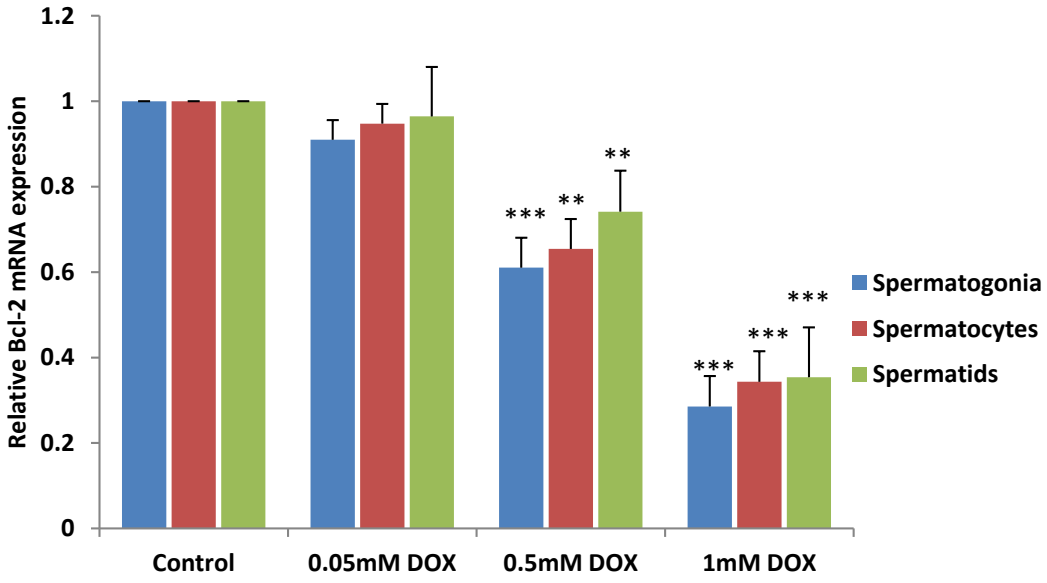

Figure 5: DNA damage induced in mice germ cells by Dox treatment in mouse spermatogonia, spermatocytes and spermatids different extents of DNA damage were shown by the OTM after treatment with Dox at 0.05, 0.5 and 1 mM. The results represent the mean ± SEM of 3 independent experiments, (ns not significant; *P <0.05; **P <0.01; *** P <0.001 when compared with the respective negative control group).

Figure 5

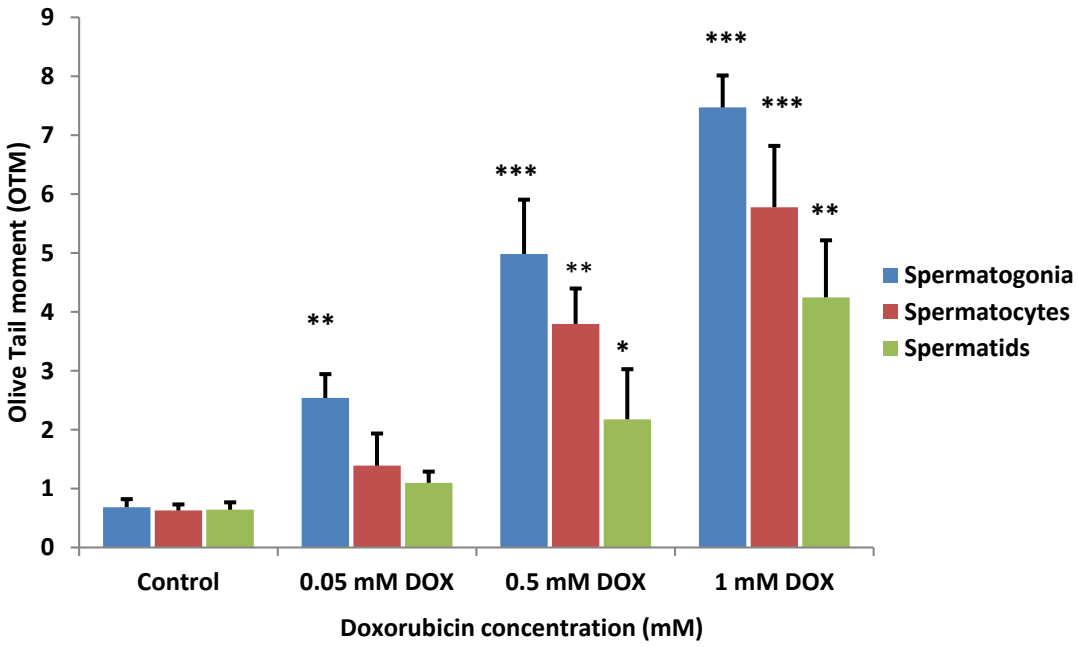

Figure 6: Comet assay results obtained from exposure of 0.05, 0.5, and 1 mM concentrations of Dox to germ cells. Comet parameters, % tail DNA were taken into account to measure DNA damage. The results are shown as mean ± SEM of 3 independent experiments, (ns not significant; *P <0.05; **P <0.01; *** P <0.001 when compared with the respective negative control group).

Figure 6

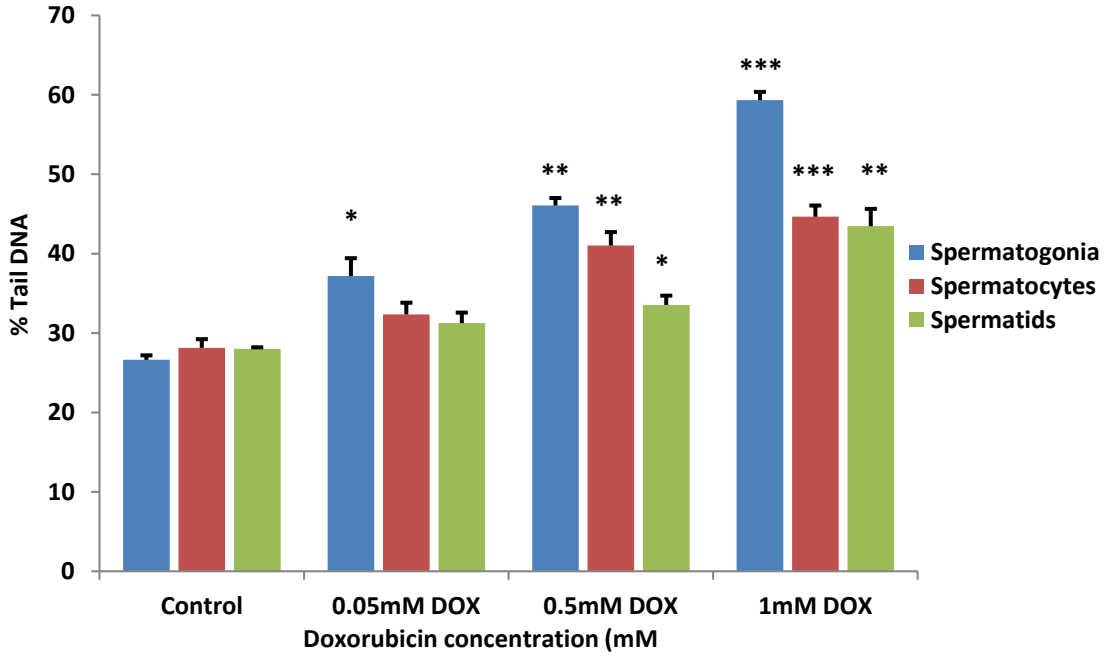

Figure 7: Effect of Dox treatment on germ cells evaluated in the TUNEL assay. Columns represent the mean percentages ± SEM of apoptotic cells for each of the three concentrations of Dox used (0, 0.05, 0.5 and 1 mM). Data were obtained from three independent experiments. Each dose level within a cell type has been compared with its respective negative control group. (ns not significant; *P <0.05; **P <0.01; *** P <0.001).