Detection of phase specificity of *in vivo* germ cell mutagens in an *in vitro* germ cell system

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Abstract

In vivo tests for male reproductive genotoxicity are time consuming, resource-intensive and their use should be minimised according to the principles of the 3Rs. Accordingly, we investigated the effects *in vitro*, of a variety of known, phase-specific germ cell mutagens, i.e. pre-meiotic, meiotic, and post-meiotic genotoxins, on rat spermatogenic cell types separated using Staput unit-gravity velocity sedimentation, evaluating DNA damage using the Comet assay. N-ethyl-N-nitrosourea (ENU), N-methyl-N-nitrosourea (MNU) (spermatogenic phase), 6-mercaptopurine (6-MP) and 5-bromo-2'-deoxy-uridine (5-BrdU) (meiotic phase), methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) (post-meiotic phase) were selected for use as they are potent male rodent, germ cell mutagens *in vivo*. DNA damage was detected directly using the Comet assay and indirectly using the TUNEL assay. Treatment of the isolated cells with ENU and MNU produced the greatest concentration-related increase in DNA damage in spermatogonia. Spermatocytes were most sensitive to 6-MP and 5-BrdU while spermatids were particularly susceptible to MMS and EMS. Increases were found when measuring both Olive tail moment (OTM) and % tail DNA, but the greatest changes were in OTM. Parallel results were found with the TUNEL assay, which showed highly significant, concentration dependent effects of all these genotoxins on spermatogonia, spermatocytes and spermatids in the same way as for DNA damage. The specific effects of these chemicals on different germ cell types matches those produced *in vivo*. This approach therefore shows potential for use in the detection of male germ cell genotoxicity and could contribute to the reduction of the use of animals in such toxicity assays.

1. Introduction

The detection and investigation of reproductive toxicants represent one of the major current challenges in toxicology because of the great number of compounds to be investigated and the difficulty of testing male germ cells at different phases of their development (Parodi *et al.*, 2015; Tralau *et al.*, 2012). A particular problem in germ cell mutagenicity studies is the relative lack of suitable tools for detecting mutation induction (Yauk *et al.*, 2015). Historically, studies have utilized huge numbers of animals in assays such as the morphological specific locus (MSL) test (Russell *et al.*, 1979) and dominant lethal assay (Anderson *et al.*, 1977), to reveal valuable information about the relative sensitivities male germ cells at different phases of development (phase-specificity). Nevertheless, there is still a general paucity of information on how endogenous factors, for example genetic polymorphisms and exogenous factors such as environmental-toxin exposure, affect the type of germ cell mutations induced and the risk of their induction (Beal *et al.*, 2012).

In animal tests the rules of the 3 R's: Reduction, Refinement and Replacement (Russell and Burch, 1959), should be applied in planning and performing experiments (Flecknell, 2002). Currently, a variety of alternative animal techniques for assessing the toxicity/genotoxicity of compounds have been developed (Jung *et al.*, 2015; Kandarova and Letasiova, 2011). STAPUT methods require far fewer animals compared with traditional methods thus aiding reduction efforts. Since the animals are not treated, this refines toxicological approaches. Therefore, because the uses of STAPUT combine these advantages, its use in a novel toxicity testing strategy could potentially replace some *in vivo* testing (Habas *et al.*, 2014). The present study is a first step in testing this idea.

There is a growing consensus that the inability to detect mutagens in human germ cells is the result of technological limitations rather than species differences between animal and human susceptibility (Beal *et al.*, 2012). Furthermore, hereditary disorders represent a major cost to health care systems (Directors, 2015). In addition, there is now longstanding public concern over the genetic consequences of lifestyle choices and environmental exposures (Chatterjee *et al.*, 2015). Therefore, there is an urgent need to refine the appropriate germ cell mutagenesis tests and deepen our understanding of germ cell mutagenesis (Beal *et al.*, 2012). Many different experimental methods are in use for investigation of genotoxicity of chemicals in animals (Parasuraman, 2011). There is, for example, increasing interest in the use of omics technologies to guide the development of biomarker panels to help predict whether a chemical would elicit a specific response under particular exposure conditions (Hartung and McBride, 2011; Wilson *et al.*, 2013).

However, a number of common chemotherapy medications have toxic consequences for spermatogenesis and can result in a drastic reduction in sperm count and quality (Dere *et al.*, 2013). Animal models have frequently been used to assess the toxicity of such drug exposures and such experiments permit researchers to study the effects on both non-target and target tissue and cell types at a level of detail which is usually not possible in humans (Dere *et al.*, 2013). In this way, safe dose levels of chemotherapeutic compounds have been determined in mouse and rat studies prior to their use in human clinical trials. Even so, the long-term effect of some of these drugs on fertility and the risk of heritable disease still remain to be clarified.

Faults in germ cell progression resulting from DNA damage can result in infertility and the transmission of genetic alterations to the offspring (Anderson *et al.*, 1999;

Brinkworth, 2000; Li *et al.*, 2014). Additionally, spermatogenic failure can occur at different levels, from defective transmigration of primordial germ cells (PGC), through spermatogenic arrest and spermatogonial stem cell losses to errors in spermiogenesis (Jan *et al.*, 2012). All or any of these complications can result in infertility as a result of azoospermia, severe oligozoospermia and asthenozoospermia or any combination of these. Indeed infertility can occur also in the absence of these in normozoospermic men whose semen parameters are nonetheless normal (Jan *et al.*, 2012). Thus, animal models are currently indispensable in many areas of reproductive research, not just reproductive genetic toxicology. The goal of the present work therefore was to explore a novel, *in vitro* alternative to *in vivo* experimentation and determine the extent to which it has the potential to yield information comparable to that already obtained in extensive, historical *in vivo* (dominant lethal) assays, an assay that for many years formed the bedrock of germline genotoxicity testing (e.g. Anderson *et al.*, 1981; Ehling *et al.*, 1978; Topham, 1980).This has been attempted for the Comet assay *in vivo* (Hartmann et al., 2013) but not yet with an *in vitro* approach.

It is well known that ENU and MNU, which are direct-acting alkylating agents (Beckwith *et al.*, 2000; Seeley and Faustman, 1995), primarily affect spermatogonia *in vivo* (Hitotsumachi *et al.*, 1985; O'Brien *et al.*, 2015). These alkylating agents have been found to induce mutagenesis by transferring an ethyl group to nucleophilic oxygen or nitrogen sites on deoxyribonucleotides, leading to base mismatch within DNA replication (Imai *et al.*, 2000; van Boxtel *et al.*, 2010). Russell et al., (1979) reported that treating male mice with ENU causes mutations that affect

the spermatogonial stem cells and they also found that the spermatogonia had the highest rate of mutation in all cell types examined. A high frequency of mutations was also found in spermatogonial cells after treatment with ENU by others (Katoh *et al.*, 1994; Provost and Short, 1994; Russell *et al.*, 2007). Previous studies showed that most of the mutations were intragenic after treatment with ENU (Marker *et al.*, 1997; Miltenberger *et al.*, 2002). In addition, mutations induced by ENU in spermatogonia, were found to be single base pair changes (Miltenberger *et al.*, 2002). MNU also has been found to be highly mutagenic in differentiating spermatogonia (Russell *et al.*, 2007; Russell and Hunsicker, 1983). Spermatogonia also showed a highly significant increase in genetic damage and apoptosis after exposure to ENU and MNU (O'Brien *et al.*, 2015). 6-MP and 5-BrdU are considered to be the major analogue drugs for therapy of acute lymphoblastic leukemia and autoimmune diseases, and have been used for four decades (Kanemitsu *et al.*, 2009; Levkoff *et al.*, 2008). 6-MP is metabolized by enzyme activity of thiopurine methyltransferase (TPMT), and therefore is anabolized by several enzymes to form 6-thioguanine nucleotides, leading to the induction of cytotoxicity as a result of its incorporation into DNA (Kanemitsu *et al.*, 2009). 6-MP has also been shown to cause chromosomal damage and aberrations in the spermatocytes of male mice (Mosesso and Palitti, 1993). It has also been shown that germ cells treated with 6-MP have the greatest response in early meiotic spermatocytes (Generoso *et al.*,

1975). The thymidine analogue 5-bromo-2′-deoxyuridine (5-BrdU) is a genotoxic compound that is incorporated into DNA, causing specific-locus mutations and inhibition of cell proliferation (Morris, 1991). 5-BrdU is a nucleoside analogue modified via halogen substitution, and its derivatives are widely used in antitumour agent studies (Kagawa *et al.*, 2008). Histological examination of cultured rat spermatocytes after injection with 5-BrdU has demonstrated that 5-BrdU mostly labels spermatocytes (Hue et al., 1998) where it causes a highly significant increase in DNA damage and apoptosis (Attia, 2012). EMS and MMS are alkylating agents that represent one of the most important classes of anticancer agents and play a major role in the treatment of several types of cancers (Chaney and Sancar, 1996; Kondo *et al.*, 2010). MMS and EMS have been studied in mature spermatozoa to examine mutagenesis in different phases of spermatogenesis in male mice (van Delft *et al.*, 1997). It has been found that EMS and MMS induce a high incidence of dominant lethal mutations in spermatids (Ehling, 1971). They have also been found to be mutagenic in the last phase of spermatogenesis, i.e. late spermatids and spermatozoa (van Delft *et al.*, 1997) and to induce chromosomal aberrations, dominant lethal mutations, and heritable translocations in these cells in mice (Ashby *et al.*, 1996; Generoso *et al.*, 1995; Russell *et al.*, 1992; Vogel and Nivard, 1997).

2. Materials and Methods

2.1. Animals

159 Sexually mature male Sprague-Dawley rats (10-12 weeks old) were obtained from
160 the Institute of Cancer Therapeutics (ICT), University of Bradford, UK where they
161 were maintained under standard conditions. All animal care procedures were carried
162 out according to the National Research Council's Guide for the Care and Use of
163 Laboratory Animals (2010).

164 2.2. Chemicals

165 N-nitroso-N-methylurea (MNU, CAS number 684-93-5), N-ethyl-N-nitrosourea ENU,
166 CAS number 759-73-9), 5-bromo-2'-deoxy-uridine (5-BrdU, CAS number 59-14-3),
167 ethyl methanesulphonate (EMS, CAS number 62-50-0) and methyl
168 methanesulphonate (MMS CAS number 66-27-3) (Sigma Chemical Ltd., Gillingham,
169 UK) were dissolved in phosphate buffered saline (pH 7.3 ± 0.2) before treatment, 6-
170 mercaptopurine (6-MP, CAS number 50-44-2) was dissolved in dimethyl sulphoxide
171 (DMSO) before treatment, and the concentrations were adjusted to (0.05 mM, 0.5
172 mM and 1.0 mM) when mixed with the cells.

173 2.3. Cell isolation and culture.

174 The method for fractionation of rat testicular germ cells was modified from that
175 described previously for the mouse (Habas *et al.*, 2014). Briefly, four testes were
176 collected from two adult Sprague-Dawley rats (10-12 weeks old), decapsulated, and
177 the seminiferous tubules placed into ice cold Dulbecco's Modified Eagle's medium
178 (DMEM), dispersed by gentle pipetting, minced and resuspended in fresh DMEM
179 containing collagenase (5mg/ml) and DNase (1µg/ml) (both from Sigma, Poole, UK),
180 then incubated at 32°C for 20 min. The cells were left to stand for 5 min before being
181 filtered through an 80µm nylon mesh (Tetco Inc., Briarcliff Manor, NY), centrifuged at
182 600 × *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, 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 immunohistochemistry and Western blotting for phase-specific markers exactly as described previously (Habas *et al.*, 2014). The viabilities of the freshly isolated spermatogonia, spermatocytes and spermatids were over 98%, as evidenced by trypan blue exclusion of these cells. Immunocytochemical analysis revealed that the range of purities of the cells from the spermatogonial fractions was 86% - 90% across the 3 independent experiments that were performed. For spermatocyte fractions it was 88% - 90% and 88% - 92% for the spermatid fractions. The results were confirmed by Western blotting (Figure1 A and B).

Germ cell preparations showed >95% viability as indicated by the trypan blue exclusion method (Phillips, 1973) at the time of plating, immediately after isolation. Morphologically normal germ cells were found by examination of cell smears stained with haematoxylin in the light microscope, and cells from each fraction were counted on the haemocytometer to determine the number of cells obtained. To determine the purity of the cell populations, slides with germ cell populations were stained by immunohistochemistry according to the manufacturer's instructions, as described previously (Habas *et al.*, 2014). After determining which fractions contained the greatest number of each cell type they were pooled and the freshly isolated germ cells were then washed with PBS and used immediately in the Comet or TUNEL

208 assays. The purity of each population was confirmed by Western blot for the
209 presence or absence of spermatogonia-, spermatocyte- and spermatid-specific
210 proteins Figure 1A and B.

211 2.4. Treatment

212 Germ cell suspensions (1.5-2.5 x 10^5 cell/ml) were mixed with fresh RPMI medium
213 (total volume 1000 µl). One hundred µl of mixed germ cells were then added to each
214 treatment tube (100 µl mixed germ cells, 890 µl RPMI medium, plus 10 µl of
215 chemical or 900 µl RPMI for the negative control). Cells were treated with different
216 concentrations (0, 0.05, 0.5, and 1mM) of chemicals at 37°C for 1 h. Solvent controls
217 were used for the 0 mM dose level. The treated and untreated germ cells were used
218 in the Comet assay and TUNEL assay.

219 *In vivo* data can be used to guide the selection of exposure levels that were used *in*
220 *vitro* according to guidance provided (Guidance, FDA 2005). However, for animal
221 cells a simple rule of thumb is mg/ml ≡ mg/Kg.bw is based on the principle that
222 organism are >90 % water.

223 2.5. Slide preparation

224 The method described by Anderson et al., (1997) was followed with some
225 modifications. One hundred µl of 1% low melting agarose (LMP) (Invitrogen, Paisley,
226 UK: 15517-022) was added to the cell pellet to create a cell suspension. The cell
227 suspension was transferred to slides pre-coated with 1% normal melting point (NMP)
228 agarose. The slides were placed on an ice block for 5 min, after which 100 µl of 0.5%
229 LMP was added on top and slides were placed on ice for 5 min. The slides then were
230 submerged in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl pH 10.0
231 containing 1% Triton X-100 and 40 mM dithiothreitol) for 1 h at room temperature

and protected from light to prevent any light-induced damage. Following this initial lysis period, proteinase K (Sigma) 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.

2.6. Unwinding and electrophoresis

The slides were placed on a horizontal gel electrophoresis platform and covered with an alkaline solution of 300 mM NaOH and 1 mM Na_2EDTA. The slides were left in the solution for 30 min at 4 °C to allow unwinding of the DNA and expression of alkali-labile sites. The power supply was set at 20 V and 300 mA. The DNA was electrophoresed for 20 min and the slides rinsed gently 3 times with 400 mM Tris (pH 7.5) to neutralize the excess alkali. Each slide was stained with 50 µl of 20µg/ml ethidium bromide (Sigma) and covered with a coverslip.

2.7. Examination of cells

Fifty cells per slide per 3 or 4 replicate experiments per treatment group were analysed at 200X viewing magnification using a fluorescent microscope (Leica, Wetzlar, Germany) 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 In the Comet assay, Olive tail moment (OTM) and % tail DNA were measured for isolated germ cells. Comet tail length is the maximum distance the damaged DNA migrates from the centre of the cell nucleus, and the OTM 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 (Kumaravel and Jha, 2006; Kumaravel *et al.*, 2009).

2.8. TUNEL staining and quantitation

DNA fragmentation was examined on the separate cell samples of the same cell populations by the modified TUNEL method first proposed by Gavrieli *et al*. (Gavrieli *et al.*, 1992) with a commercial apoptosis detection kit (Terminal Deoxynucleotidyl Transferase Detection Kit; Promega, UK, Ltd). In brief, the coverslips were 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; 0.3 U/µl terminal deoxynucleotidyl transferase [TdT]; all from Sigma) for 60 min (humidity chamber, 37°C) and then washed twice in PBS. (H_2O_2-blocking of endogenous peroxidases was not performed as the testis is low in peroxidases so it is rarely necessary.) After multiple washing steps, the cells were treated with 2% v/v Extravidin peroxidase in TBS with 0.1% w/v BSA for 30 min (humidity chamber, 37°C), rinsed with PBS, and visualised by adding 3, 3-diaminobenzine (DAB) for 10 min at room temperature. Cells were washed in phosphate buffered saline (PBS); each section was counterstained with haematoxylin then examined and photographed under an Olympus CKX31 microscope (Olympus, Southend on Sea, UK). For the negative controls, sections were incubated with the reaction mix without TdT instead of the full TUNEL reaction mixture. To quantitate the incidence of apoptosis, the three types of isolated testicular germ cells were evaluated for morphology and staining, The following findings were considered to represent apoptosis: marked condensation of chromatin and cytoplasm clearly staining strongly brown or brown/black; The TUNEL-positive cells were scored in several fields on each coverslip to yield a total of at least 100 cells under a 40x objective of an Olympus CKX31 microscope. Values represent percentages from at least 100 cells from each culture.

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.

3. Results

3.1. Effect of ENU and MNU treatment

MNU and ENU displayed a concentration-response curve in both *in vitro* assays used for this investigation. The lowest doses that induced a statistically significant increase in genetic damage were 0.05 mM ENU and 0.05 mM MNU for DNA damage and 0.05 mM ENU or 0.05 mM MNU for apoptosis. In spermatogonia, both the nitrosoureas showed highly increased OTM, % tail DNA and % of apoptotic cells (Table 1). They were thus clearly much more potent as spermatogonial mutagens than 6-MP and 5-BrdU or the methanesulphonates (Table 1). The response to ENU and MNU treatment was also different between the three types of cells analysed: spermatogonia, showed the highest levels of DNA damage and apoptosis in response to these genotoxins in comparison to the other types of cells. Spermatogonial cells showed a clear concentration dependent, statistically significant increase in comet parameters and apoptosis from the concentration of 0.05 mM upwards (Figures 2 and 4) whereas the other compounds showed lower means at each dose with generally lower levels of significance and only significant at all from 0.5 mM upwards.

3.2. Effect of 6-MP and 5-BrdU treatment

6-MP and 5-Br-dU showed a concentration response-curve in both assays used for this study. The lowest doses that induced a statistically significant increase in genetic damage were 0.05 mM 6-MP and 0.05 mM 5-BrdU for DNA damage, and 0.05 mM 6-MP and 0.05 mM 5-BrdU for apoptosis. In spermatocytes, both 6-MP and 5-BrdU showed highly increased OTM, % tail DNA and the percentage of apoptotic cells. They were therefore clearly much more potent spermatocyte mutagens than MNU and ENU or the methanesulphonates (Table 2). The response to 6-MP and 5-BrdU treatment was also different for the three types of cells analysed with spermatocytes, showing the highest levels of DNA damage and apoptosis in response to 6-MP and 5-BrdU treatment. Spermatocytes showed a clear concentration-dependent, statistically significant increase in comet parameters and apoptosis from the concentration of 0.05 mM upwards (Figure 2, 3 and 4) whereas the other compounds showed lower means at each dose with generally lower levels of significance and only significant from 0.5 mM upwards.

3.3. Effect of EMS and MMS treatment

MMS and EMS also showed a concentration-response curve in both assays used for this study. The lowest concentrations that induced a statistically significant increase in genetic damage were 0.05 mM MMS or 0.05 mM EMS for DNA damage and 0.05 mM MMS or 0.05 mM EMS for apoptosis. In spermatids, both the methanesulphonates MMS and EMS showed highly increased OTM, % tail DNA and the % of apoptotic cells. They were therefore clearly much more potent spermatid mutagens than MNU and ENU or 6-MP and 5-BrdU (Figure 4). The response to MMS and EMS treatment was also different for the three types of cells analysed: spermatids, which showed the highest levels of DNA damage and apoptosis in response to MMS and EMS treatment than the other types of cells. Spermatocytes

showed a clear concentration-dependent, statistically significant, increase in comet parameters and apoptosis from the concentration of 0.05 mM upwards (Figures 2, 3 and 4) whereas the other compounds showed lower means at each dose with generally lower levels of significance and only significant from 0.5 mM upwards. The values of various Comet measurements as quantified with Comet 6.0 software generated from MMS and EMS studies are given in Table 3. The results showed that OTM and %Tail DNA gave good correlations with the concentration of MMS and EMS.

4. Discussion

The purpose of this study was to explore the potential for our recently developed *in vitro* male germ cell test system (Habas *et al.*, 2014) to detect male germ cell genotoxins. Furthermore, this type of study could extend our understanding of how rodent germ cells could be used for monitoring developmental toxicity/genotoxicity and its relevance to spermatogenic processes. Compositional and metabolic differences between different types of male germ cells lead to differing susceptibilities to genotoxicity and mutation induction so the careful analysis of such phase specificity is important. This can yield valuable information about the potential mechanisms involved in the toxicity and thus increase the significance of the findings. This is especially important since mutations induced in the germline can affect not only the exposed generation but also an unlimited number of generations thereafter (Verhofstad *et al.*, 2008).The most well-established techniques that have historically been used to demonstrate that germline mutations appear in the next and subsequent generations are all very animal-intensive. These include the MSL test and the dominant lethal test (Verhofstad *et al.*, 2008). In addition, these approaches have relatively low sensitivity, and are time consuming and expensive (Verhofstad *et*

353 *al*., 2008). Nevertheless, these approaches have played a major role in genotoxicity
354 assessment and in particular, in establishing precise mutation rates associated with
355 specific exposure levels (MacGregor *et al.*, 2015).

356 To reduce the number of *in vivo* assays that detect effects in germ cells and to
357 adhere to the principles of the 3Rs, *in vitro* or *ex vivo* tests should be performed first
358 (Ouedraogo *et al.*, 2012; Verhofstad *et al.*, 2008).Currently, a variety of alternative
359 animal techniques for assessing the toxicity/genotoxicity of compounds have been
360 developed (Jung *et al.*, 2015; Kandarova and Letasiova, 2011). A number of *in vitro*
361 assays are currently available such as the detection of chromosome damage or
362 sister-chromatid exchanges in primary cultures of spermatogonia or spermatocytes
363 (Perrin *et al.*, 2007). However, these involve low-frequency end-points and are not
364 suitable for the rapid screening of chemicals for genotoxicity or mutagenicity.
365 Furthermore, even though it is possible to culture such cells in the presence of
366 Sertoli cells, there is doubt about how well these germ cells reflect the *in vivo*
367 situation, given that they do not have the intimate, all-enveloping contact with the
368 Sertoli cells found in the seminiferous tubule. The use of cultured Sertoli cells with
369 testicular germ cells has in fact been used with some success in the past (e.g., (Gray
370 and Beamand, 1984). However, it has not gained widespread applicability mainly
371 because of difficulty of reproducing *in vitro* the tight junctions between Sertoli cells
372 that form the blood-testis barrier. We have sought to avoid these problems by
373 culturing the cells for the shortest possible period and to turn the absence of Sertoli
374 cells (and hence the more direct exposure to chemicals that this affords) into an
375 advantage by using the system to detect reproductive hazards, rather than for strict
376 risk assessment.

Accordingly, we examined DNA-strand breakage and the induction of apoptosis in short-term, primary cultures of testicular germ cells enriched for spermatogonia, spermatocytes and spermatids. These were tested using 6 chemicals whose reproductive genotoxicity is well established and which show clear cell-type specificity *in vivo*. Genotoxicity assessment after exposure to ENU, MNU, 6-MP, 5-BrdU and, MMS or EMS was conducted using the alkali version of the Comet assay to detect DNA strand breaks and the TUNEL assay to detect apoptosis. Spermatogonia were the most sensitive to ENU and MNU; spermatocytes were most sensitive to 6-MP and 5-BrdU while spermatids were the most sensitive cell type to MMS and EMS. Crucially, all these results match the positive results found *in vivo* and the cell-type specificity of each chemical found *in vivo* was the same as that which we found with our *in vitro* system. Parallel results were found using the TUNEL assay, which also showed highly significant, concentration-dependent effects of these 6 genotoxins on spermatogonia, spermatocytes and spermatids in the same way as for DNA damage.

In the present study it would appear that the *in vitro* – *in vivo* correlation (IVIVC) is working such that the *in vitro* concentrations and in vivo absorption directly reflect a proportional, linear relationship between *in vitro* (dissolution) and *in vivo* (fraction absorbed). This IVIVC has been assumed in all *in vitro* to *in vivo* calculations used in our laboratory and in all cases; the results have revealed a similarly directly proportional, linear IVIVC for all our *in vitro* to *in vivo* extrapolations (e.g., Anderson *et al.*, 2003). This implies that in our hands, the *in vitro* and *in vivo* systems operate under comparable kinetics and toxicodynamics.

The results for ENU and MNU are similar to findings by Russell, Hunsicker and Russell (2007), who also found genetic damage in spermatogonia when treated with

ENU and MNU. In the present study, comparing the chemicals for the endpoints studied (i.e., Comet assay and TUNEL assay) revealed that ENU and MNU are much more effective on spermatogonia than spermatocytes and spermatids. These results suggest that both chemicals are highly genotoxic in differentiating spermatogonia, in agreement with *in vivo* data published previously (Guenet, 2004; Russell et al., 2007; Russell and Hunsicker, 1983; Siepka and Takahashi, 2005). In addition, increased spontaneous frequency of gene mutations and chromosome damage has more recently been reported in these cells (O'Brien *et al.*, 2013).

A similar trend was observed in spermatocytes, which showed a highly significant increase in DNA damage and apoptosis after exposure to 6-MP or 5-BrdU. This increase was dramatically greater than in spermatids or spermatogonia. Thus, spermatocytes are the most sensitive to 6-MP and 5-BrdU in agreement with the findings *in vivo* that showed 6-MP to be a potent chemical for inducing DNA damage in spermatocytes (Mosesso and Palitti, 1993) along with 5-BrdU (Perrard *et al.*, 2003). Similarly, Witt and Bishop (1996) have shown that 6-MP induced dominant lethal mutations specifically in spermatocytes (Witt and Bishop, 1996).

Spermatids were found to be the most sensitive to MMS and EMS in the induction of genetic damage and apoptosis in agreement with *in vivo* studies that showed a decrease in the number of spermatids and a high frequency of chromosome aberrations induced by these (and other) methanesulphonates in spermatids (Kuriyama *et al.*, 2005; Matsuda *et al.*, 1989).

Our work with these chemicals also suggests that OTM and percent tail DNA give good correlations with the concentration of genotoxic agents used. Statistically, our results did not find much difference between OTM and % tail DNA. The close

concordance between the Comet assay and TUNEL assay results is remarkable. Both assays detect DNA strand breakage but whereas the Comet assay reports breakage caused by exogenous agents, the TUNEL assay shows strand breaks induced by endogenous nucleases as part of a cellular suicide mechanism. Such cellular suicide can be induced by events such as severe DNA-strand breakage so the results demonstrate effects on two different end-points of genotoxicity, one direct and one indirect. This demonstrates that multiple end-points can be utilised in our short-term culture system, even when one is consequent upon another, showing that the system is flexible enough to be used for mechanistic studies as well as hazard detection.

The ease and consistency with which our short-term culture system coupled with the comet and TUNEL assays has detected DNA damage and apoptosis in male rat germ cells suggests it could have considerable utility for the identification of male reproductive genotoxins. This is despite the fact that the cells are cultured in the absence of Sertoli cells. However, it may be that in the absence of any protection from either the blood-testis barrier or the detoxifying abilities of Sertoli cells, the germ cells are rendered more susceptible to toxins, thus producing a more sensitive assay for reproductive genotoxic potential.

5. Conclusion

The level of DNA damage *in vitro* is dependent on both the gentoxicant and the type of germ cell, and occurs at concentrations known to be relevant to testicular and reproductive toxicity *in vivo*. Furthermore, these results indicate that Staput-isolated rat testicular germ cells provide a suitable model *in vitro* to study DNA damage in different phases of spermatogenesis. The high concordance between the existing *in*

vivo data and the present results indicate that this approach should be further investigated for its potential to detect genetic effects in reproductive cells *in vitro* with the long-term aim of enabling a reduction in animal testing for reproductive genotoxins.

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7. Conflict of Interest Statement

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9. Figure legends

Figure 1. Western blot analysis of Staput-purified germ cells using antibodies for specific cell-type markers. Figure 1A: Anti-glial cell line-derived neurotrophic factor receptor (GDNFR) antibody was used to detect spermatogonia (panel B); anti-synaptonemal complex protein 3 (SCP-3) antibody was used to detect spermatocytes (panel C); anti-transition protein 1 (TP1) antibody (panel D), and the protein loading control GAPDH is shown in panel A. The relative expression levels of GDNFR, SPC3 and TP1 were expressed as GDNFR, SCP-3 and TP1/ GAPDH ratios. Results are the mean ± SEM from four independent experiments. ***p< 0.001.

Figure 1B: Comparison of GDNFR, SCP-3 and TP1 expression in isolated testicular germ cells. Spermatogonia, spermatocytes, and spermatids were examined by Western blot analysis. The density of each band was quantified by Image 1.45 software (arbitrary units) and the relative expression levels of GDNFR, SCP-3 and TP1 was measured by GDNFR, SCP3 and TP1 / GAPDH ratios. Results are the mean ± SEM. from four independent experiments. ***p < 0.001.

Figure 2. Induced DNA damage in germ cells in the Comet assay after treatment with ENU, MNU, 6-MP, 5-BrdU, MMS and EMS at different concentrations (0.05 mM, 0.5 mM and 1 mM) for 1h. OTM was used for DNA damage quantification. Data represent the means ± SE obtained from three independent experiments. *P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group.

Figure 3. Induced DNA damage in germ cells in the Comet assay after treatment with ENU, MNU, 6MP, 5BrdU, MMS and EMS at different concentrations (0.05 mM, 0.5 mM and 1 mM) for 1h. %Tail DNA was used for DNA damage quantification. Data represent the means ± SE obtained from three independent experiments. *P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group.

Figure 4. Effect of ENU, MNU, 6MP, 5BrdU, MMS and EMS 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 ENU, MNU, 6-MP, 5-BrdU, MMS and EMS used (0.05 mM, 0.5 mM and 1 mM). Data represent the means ± SE obtained from three independent experiments. *P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group.

720 Table 1. Individual data for the effects of ENU and MNU on isolated germ cells
721 measured by the Comet and TUNEL assays. Comet parameters: OTM and % tail
722 DNA; TUNEL parameter: percentage of apoptotic cells. Data shown represent group
723 values (mean ± SEM) of three experiments (100 cells scored per experiment). ns not
724 significant, *p <0.05, **p <0.01 and *** p <0.001 versus control.

Germ cells	OTM	(%)Tail DNA	Apoptotic cells (%)
Spermatogonia			
Control	1.23 ± 0.06	9.19 ± 0.06	8.00 ± 0.58
0.05mM ENU	2.33 ± 0.13**	11.45± 0.37**	13.67±0.33**
0.5mM ENU	6.21± 0.49 ***	20.64 ± 0.52***	35.67±0.33***
1mM ENU	9.29 ± 0.20***	28.38 ± 0.81***	49.00±0.58***
Control	1.03 ± 0.08	6.23 ± 0.33	7.33 ± 0.33
0.05mM MNU	2.09 ±0.21*	6.86 ± 0.42*	9.00 ± 0.58*
0.5mM MNU	5.87 ± 0.65**	20.29±1.51**	16.33 ± 1.20**
1mM MNU	8.34 ± 0.28***	25.10± 0.67**	37.67 ± 1.20***
Spermatocytes			
Control	0.91 ± 0.08	3.58 ± 0.36	7.00 ± 0.58
0.05mM ENU	1.35 ± 0.23 ns	5.74 ± 0.69 ns	10.00 ± 0.58 ns
0.5mM ENU	3.01 ± 0.15 *	10.71± 1.13 *	12.67 ± 0.88 *
1mM ENU	3.78 ± 0.21**	14.40 ± 1.12**	19.67 ± 0.67**
Control	0.72 ± 0.09	3.97 ± 0.37	7.00 ± 0.58
0.05mM MNU	1.19 ± 0.20 ns	6.28 ± 0.55	8.33 ± 0.88
0.5mM MNU	2.59 ± 0.53 *	11.73 ±1.16	11.00 ± 0.58
1mM MNU	3.94 ± 0.43**	14.45 ±1.18	17.00 ± 0.58
Spermatids			
Control	0.59 ± 0.05	2.77 ± 0.31	6.67 ± 0.33
0.05mM ENU	0.75 ± 0.09 ns	5.46 ± 0.95 ns	8.67 ± 0.33 ns
0.5mM ENU	1.87 ± 0.37 *	6.24 ± 0.92 *	11.00 ± 0.58*
1mM ENU	2.66 ± 0.28**	11.71± 1.20 **	17.67 ± 0.88**
Control	0.79 ± 0.08	3.50 ± 0.35	6.67 ± 0.67
0.05mM MNU	1.64 ± 0.20 ns	6.15 ± 0.61ns	8.67 ± 1.20 ns
0.5mM MNU	2.11± 0.24 *	8.82 ± 0.83 *	12.00 ± 0.58 *
1mM MNU	3.02 ± 0.28**	12.46 ±0.84**	17.33 ±0.88**

725

726

727 Table 2. Individual data for the effects of 6-MP and 5-BrdU on isolated germ cells
728 measured by the Comet and TUNEL assays. Comet parameters: OTM and % tail
729 DNA; TUNEL parameter: percentage of apoptotic cells. Data shown represent group
730 values (mean ± SEM) of three experiments (100 cells scored per experiment). ns not
731 significant, *p <0.05, **p <0.01 and *** p <0.001 versus control.

Germ cells	OTM	(%)Tail DNA	Apoptotic cells (%)
Spermatogonia			
Control	0.80 ± 0.06	3.35 ± 0.33	8.38 ± 0.72
0.05mM 6-MP	1.20 ± 0.10ns	4.31± 0.30 ns	10.52 ± 1.56 ns
0.5mM 6-MP	2.63 ± 0.48ns	7.20 ± 0.70 ns	11.14 ± 0.50ns
1mM 6-MP	3.53 ± 0.38**	11.31± 0.64**	19.73 ±1.43**
Control	0.80 ± 0.04	3.59 ± 0.13	7.67 ± 0.33
0.05mM 5-BrdU	1.50 ±0.20ns	6.56 ± 0.89ns	11.00 ± 1.15ns
0.5mM 5-BrdU	1.88 ± 0.16*	7.66 ± 0.65*	14.33 ± 0.88*
1mM 5-BrdU	2.80 ± 0.23**	10.34 ± 1.01**	19.00 ± 1.15**
Spermatocytes			
Control	0.87 ±0.06	3.91± 0.08	7.97 ± 0.50
0.05mM 6-MP	2.22 ±0.15*	8.50 ±0.67 *	18.11 ± 1.69 *
0.5mM 6-MP	5.63 ±0.55 **	12.41 ±0.88 **	23.47 ± 1.44**
1mM 6-MP	7.63 ±0.36 ***	19.04 ±0.93 ***	35.33 ± 1.38***
Control	0.69 ± 0.04	3.71± 0.35	7.33 ± 0.33
0.05mM 5-BrdU	1.91± 0.22*	9.36 ± 0.63*	17.00± 1.73*
0.5mM 5-BrdU	3.88 ± 0.36**	13.09 ± 0.66**	19.00 ± 1.15**
1mM 5-BrdU	6.55 ± 0.29***	16.77± 0.79***	30.66± 1.37***
Spermatids			
Control	0.75 ± 0.03	3.81 ± 0.17	6.19 ± 0.58
0.05mM 6-MP	0.98 ± 0.28ns	5.58 ± 0.90ns	8.21 ± 0.62 ns
0.5mM 6-MP	1.24 ± 0.13ns	5.87 ± 0.48ns	12.52 ± 1.28 ns
1mM 6-MP	2.45 ±0.26**	8.60 ± 0.38**	16.27 ± 1.01**
Control	0.78 ± 0.06	3.69 ± 0.34	5.00 ± 0.58
0.05mM 5-BrdU	1.43 ± 0.29ns	5.77 ± 0.88 ns	7.00 ± 0.58 ns
0.5mM 5-BrdU	1.62 ± 0.21*	7.43 ± 1.11*	11.00 ±1.15 *
1mM 5-BrdU	2.60 ± 0.16**	10.09 ± 0.61**	15.33 ± 0.88**

732

733

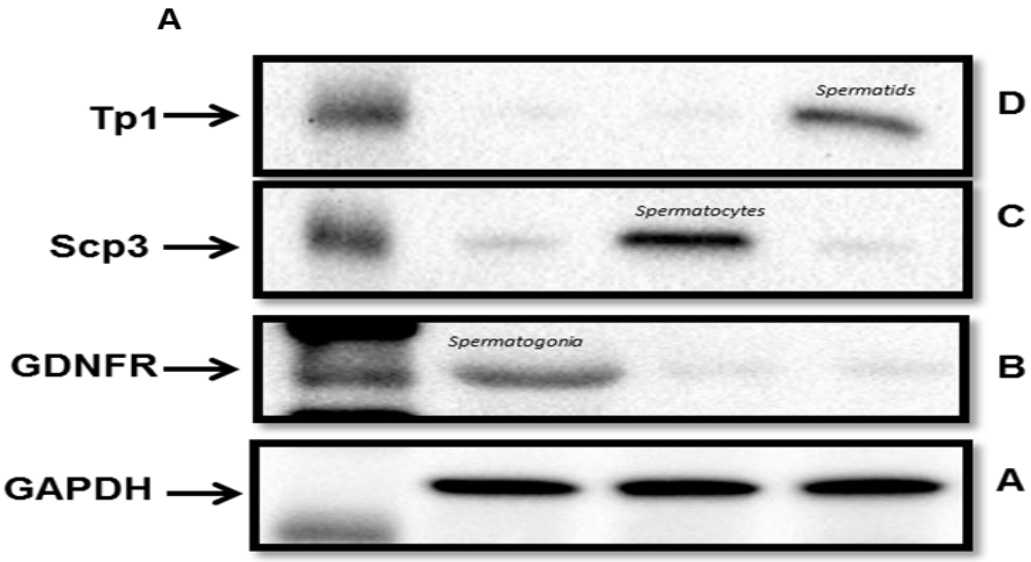
734 Table 3. Individual data for the effects of MMS and EMS on isolated germ cells
735 measured by the Comet and TUNEL assays. Comet parameters: OTM and % tail
736 DNA; TUNEL parameter: percentage of apoptotic cells. Data shown represent group
737 values (mean ± SEM) of three experiments (100 cells scored per experiment). ns not
738 significant, *p <0.05, **p <0.01 and *** p <0.001 versus control.

739

Germ cells	OTM	(%)Tail DNA	Apoptotic cells (%)
Spermatogonia			
Control	0.92 ± 0.06	3.96 ± 0.24	8.33 ± 0.33
0.05mM MMS	1.69 ± 0.19ns	6.98 ± 1.26ns	11.00 ± 0.58ns
0.5mM MMS	2.98 ± 0.53*	9.95 ± 1.37*	13.67 ± 0.6*
1mM MMS	4.91 ± 0.48**	16.96 ± 2.04**	21.00 ±1.53**
Control	1.11 ± 0.16	3.99 ± 0.38	7.33 ± 0.33
0.05mM EMS	1.37 ± 0.35 ns	6.15 ± 0.99ns	10.33 ± 0.88ns
0.5mM EMS	2.95 ± 0.21*	8.87 ± 0.92*	12.33 ± 0.88*
1mM EMS	4.36 ± 0.31**	14.09 ± 1.17**	20.33 ±1.33**
Spermatocytes			
Control	1.03 ± 0.13	4.59 ± 0.46	7.67 ± 0.67
0.05mM MMS	1.77 ± 0.09ns	9.35 ± 0.88 ns	11.33 ± 0.88 ns
0.5mM MMS	4.32 ± 0.41*	10.93 ± 0.97*	17.33 ± 0.88*
1mM MMS	6.15 ± 0.54**	18.36 ± 1.15**	22.00 ± 1.53**
Control	0.90 ± 0.09	4.12 ± 0.25	8.00 ± 0.58
0.05mM EMS	2.32 ± 0.31ns	9.12 ± 1.18ns	11.33 ± 0.67ns
0.5mM EMS	3.44 ± 0.49*	11.41 ± 0.91*	16.67 ± 0.88*
1mM EMS	5.17 ± 0.42**	21.81 ± 1.73**	20.67± 0.88**
Spermatids			
Control	0.89 ± 0.06	4.58± 0.37	7.67 ± 0.67
0.05mM MMS	2.65 ± 0.35*	12.34± 0.86*	14.00 ± 0.58*
0.5mM MMS	8.02 ± 0.76**	25.44± 0.94**	27.67 ± 1.20**
1mM MMS	11.66 ± 1.04***	36.63 ±0.75***	35.67 ± 0.88***
Control	0.92± 0.03	4.58 ± 0.22	8.00 ± 1.00
0.05mM EMS	3.04 ±0.32*	11.06 ± 1.04*	14.00 ± 0.58 *
0.5mM EMS	6.80 ±0.85**	24.49 ± 2.10**	20.33 ± 1.86**
1mM EMS	9.89 ±0.36***	33.64 ± 0.76***	34.00± 1.15***

740

741



742

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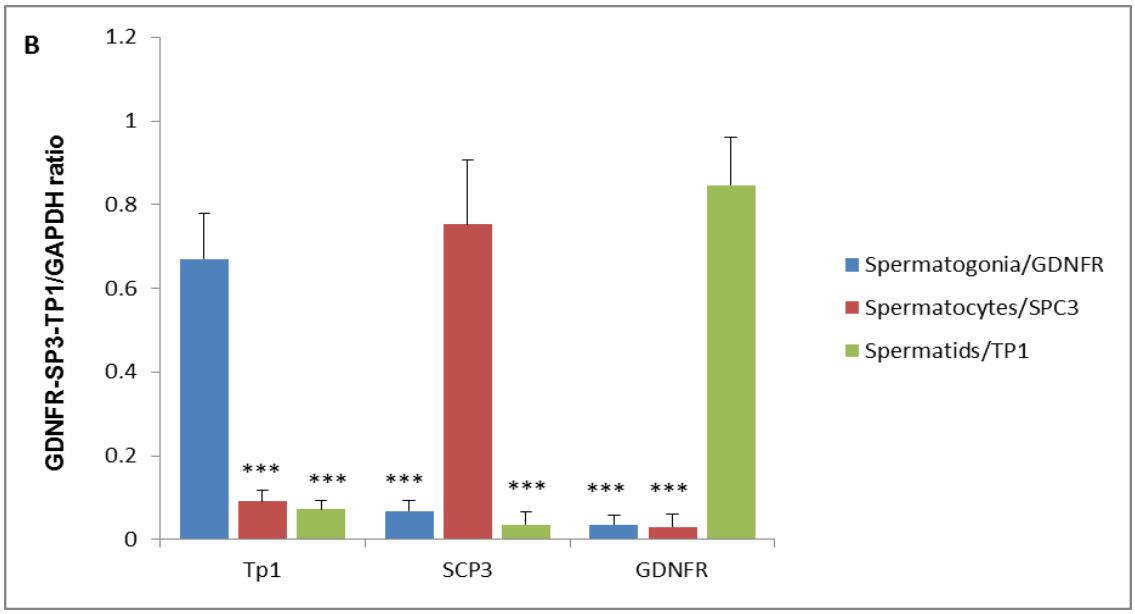

744

745

746

747 Figure 1

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749

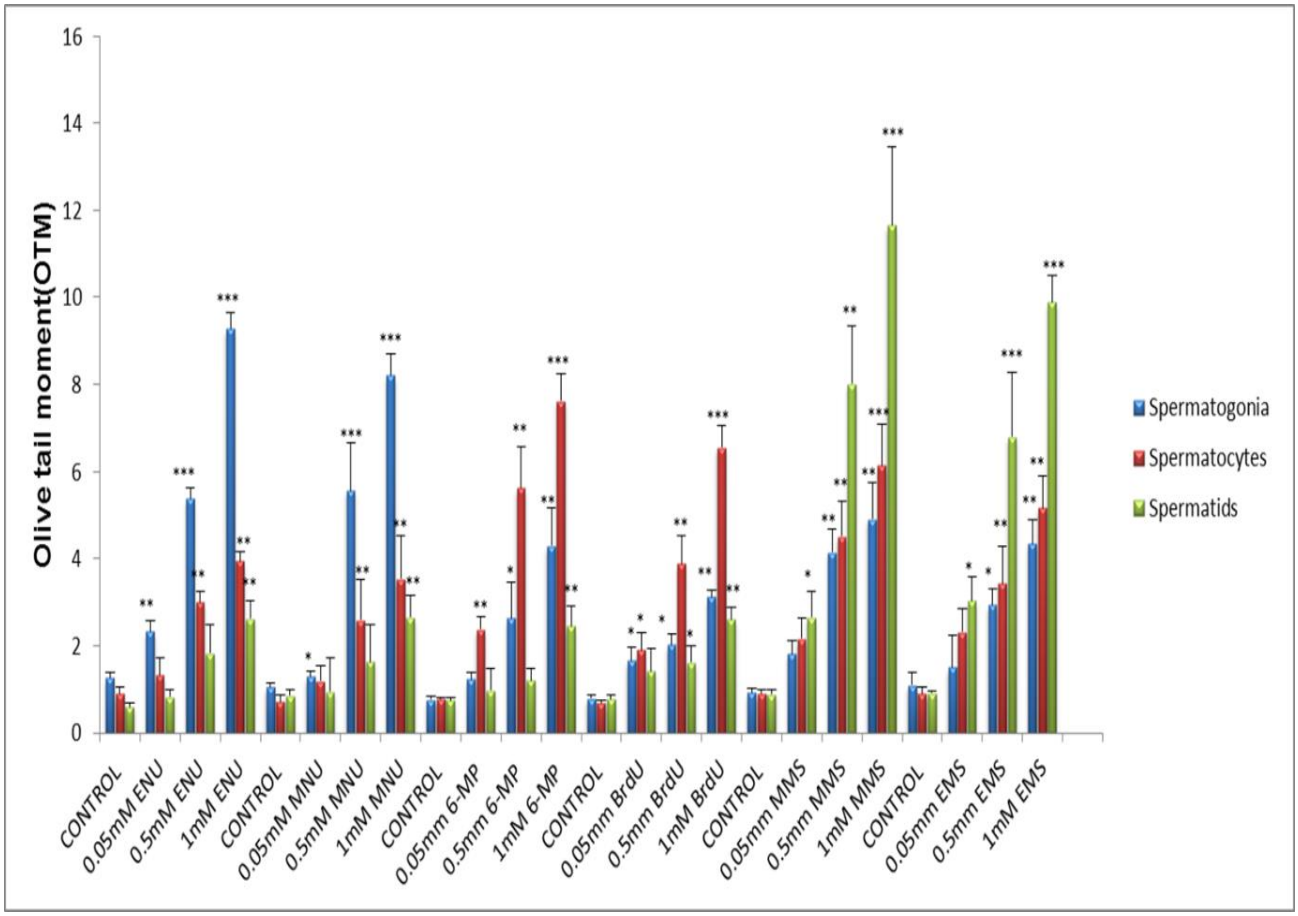
Olive tail moment(OTM)
16
14
12
10
8
6
4
2
0
CONTROL
0.05mM ENU
0.5mM ENU
1mM ENU
CONTROL
0.05mM MNU
0.5mM MNU
1mM MNU
CONTROL
0.05mm 6-MP
0.5mm 6-MP
1mM 6-MP
CONTROL
0.05mm BrdU
0.5mm BrdU
1mM BrdU
CONTROL
0.05mm MMS
0.5mm MMS
1mM MMS
CONTROL
0.05mm EMS
0.5mm EMS
1mM EMS
Spermatogonia
Spermatocytes
Spermatids

Figure 2

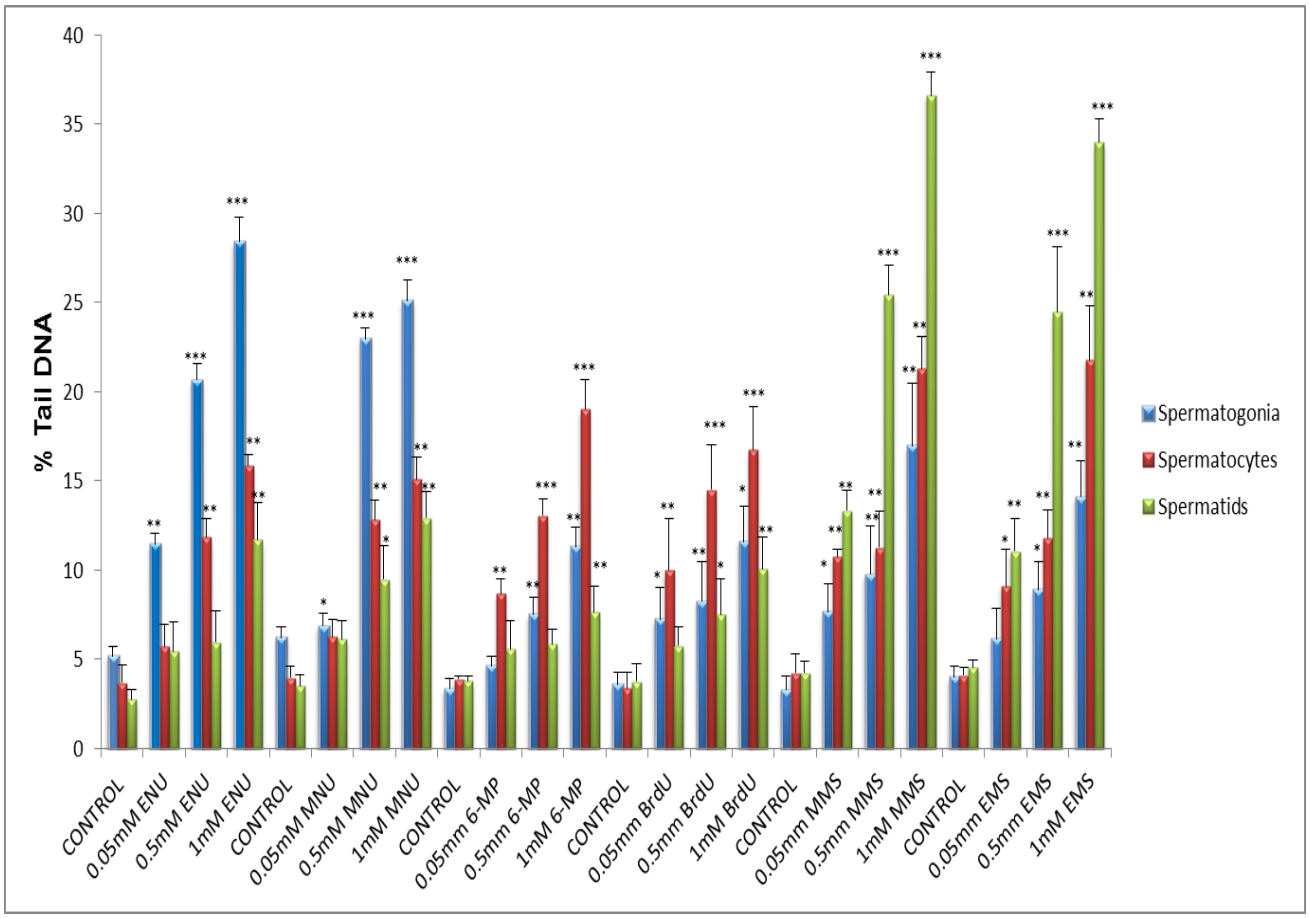
% Tail DNA
40
35
30
25
20
15
10
5
0
Spermatogonia
Spermatocytes
Spermatids
CONTROL
0.05mM ENU
0.5mM ENU
1mM ENU
CONTROL
0.05mM MNU
0.5mM MNU
1mM MNU
CONTROL
0.05mm 6-MP
0.5mm 6-MP
1mM 6-MP
CONTROL
0.05mm BrdU
0.5mm BrdU
1mM BrdU
CONTROL
0.05mm MMS
0.5mm MMS
1mM MMS
CONTROL
0.05mm EMS
0.5mm EMS
1mM EMS

Figure 3

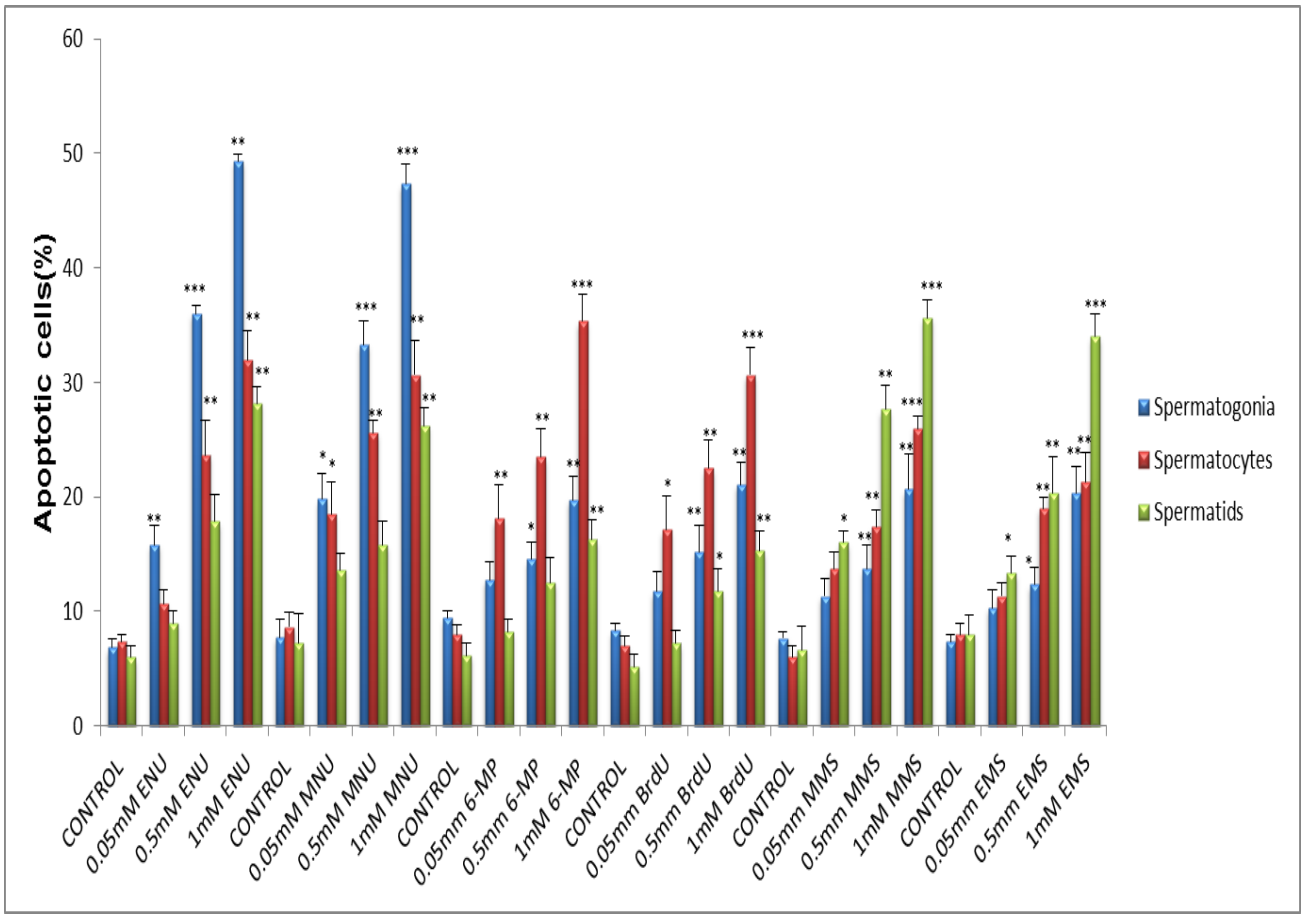
Apoptotic cells(%)
60
50
40
30
20
10
0
Spermatogonia
Spermatocytes
Spermatids
CONTROL
0.05mM ENU
0.5mM ENU
1mM ENU
CONTROL
0.05mM MNU
0.5mM MNU
1mM MNU
CONTROL
0.05mm 6-MP
0.5mm 6-MP
1mM 6-MP
CONTROL
0.05mm BrdU
0.5mm BrdU
1mM BrdU
CONTROL
0.05mm MMS
0.5mm MMS
1mM MMS
CONTROL
0.05mm EMS
0.5mm EMS
1mM EMS

Figure 4