In Vitro Responses to Known *in Vivo* Genotoxic Agents in Mouse Germ Cells

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Abstract

Genotoxic compounds have induced DNA damage in male germ cells and have been associated with adverse clinical outcomes including enhanced risks for maternal, paternal and offspring health. DNA strand breaks represent a great threat to the genomic integrity of germ cells. Such integrity is essential to maintain spermatogenesis and prevent reproduction failure. The Comet assay results revealed that the incubation of isolated germ cells with n-ethyl-n-nitrosourea (ENU), 6-mercaptopurine (6-MP) and methyl methanesulfonate (MMS) led to increase in length of Olive tail moment and % tail DNA when compared with the untreated control cells and these effects were concentration-dependent. All compounds were significantly genotoxic in cultured germ cells. Exposure of isolated germ cells to ENU produced the highest concentration-related increase in both DNA damage and gene expression changes in spermatogonia. Spermatocytes were most sensitive to 6-MP, with DNA damage and gene expression changes while spermatids were particularly susceptible to MMS. Real-time PCR results showed that the mRNA level expression of *p53* increased and *bcl-2* decreased significantly with the increasing ENU, 6-MP and MMS concentrations in spermatogonia, spermatocytes and spermatids respectively for 24 h. Both are gene targets for DNA damage response and apoptosis. These observations may help explain the cell alterations caused by ENU, 6-MP and MMS in spermatogonia, spermatocytes and spermatids. Taken together, ENU, 6-MP and MMS induced DNA damage and decreased apoptosis associated gene expression in the germ cells *in vitro*.

Introduction

Spermatogenesis is a highly organized and a complex process that is characterized by stem-cell renewal, reorganization, genome repackaging and production of differentiated daughter cells to provide a continual supply of spermatozoa [Oatley et al. 2004]. Defects or delay at all stages of the spermatogenesis process lead to infertility, but few of them can be modelled *in vitro* or in cell culture. Targeted mutagenesis in mice offers great tools to investigate these stages, and study the impact of gene function on specific stages and thus has provided novel insights into the origins of male infertility [Cooke and Saunders 2002; Yauk et al. 2015]. Animal models have also been extensively used to study normal spermatogenesis and have revealed various critical molecular mechanisms that determine whether genetic damage persists in the germline [Jan et al. 2012]. Alkylating agents affect the mammalian genome by forming DNA lesions, and thus causing base substitution mutations, or preventing DNA replication [Imai et al. 2000; van Boxtel et al. 2010]. However, ENU has been shown to be a potent alkylating agent inducing germ cell line mutation due to its strength and preferential activity in spermatogonial stem cells [Caignard et al. 2014]. It has also been shown that germ cells treated with 6-MP have the greatest response in early meiotic spermatocytes [Generoso et al. 1975; Norgard et al. 2016]. MMS has also been found to induce a high incidence of dominant lethal mutations in spermatids [Ehling 1971]. Our previous study [Habas et al. 2016], using the Comet assay in rat, also showed that ENU produced a high level of DNA damage in spermatogonia incurring significantly greater DNA damage. Spermatocytes were most sensitive to 6-MP while spermatids were particularly susceptible to MMS [Habas et al. 2016]. This present study aimed to assess the

phase specificity of the susceptibility of spermiogenic germ cells in mice to genetic damage induced by ENU, 6-MP and MMS *in vitro*.

Materials and Methods

Animals

Male adult National Medical Research Institute (NMRI) mice (10-12 weeks old; weighing 25–30 g), derived from the original stocks obtained from the Institute of Cancer Therapeutics Laboratories, were maintained under standard conditions with free access to food and water at the Animal Facility of the University of Bradford, UK. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

Cell Isolation and Culture.

The method for isolation of testicular germ cells was described previously for the mouse [Habas et al. 2014]. Briefly, six testes were collected from three adult NMRI mice (10-12 weeks old), 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) (both from Sigma, Poole, UK), then incubated at 32°C for 20 min. The cells were left to stand for 5 min before being filtered through an 80µm 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 bovine serum albumin (BSA) (Sigma, Poole, UK) was then generated below the cells, which were allowed to sediment for a standard period of 2.5 h before 31, 12 ml 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 were collected by low-speed centrifugation and resuspended in DMEM.

Isolation and morphological characteristics of mouse spermatogonia, spermatocytes and spermatids

The identity and purity of all cell preparations used in the experiments were confirmed by immunohistochemistry 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 87% − 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 also were confirmed by Western blotting for phase-specific markers exactly as described previously [Habas et al. 2016].

Treatment of Isolated Germ Cells with Chemicals

Germ cell suspensions (1.5 − 2.5 × 10^5 cell/ml) were suspended in 1 ml sterile fresh RPMI medium. One hundred µl of mixed germ cells were then added to each treatment tube (100 µl mixed germ cells, 890 µl RPMI medium, plus 10 µl of chemical or 900 µl RPMI for the negative control). Cells were treated with ENU, 6-MP and MMS at a final concentration of (0, 0.05, 0.5, and 1 mM) and all samples were incubated for 1 h at 37°C, 5% CO_2. Solvent controls were used for the 0 mM dose level. Therefore, due to the length of exposure used in the current study (1h for the Comet assay and 24 h for qPCR assay), the concentrations of ENU, 6-MP and

MMS were adjusted to the 0.05 mM – 1 mM range to ensure cell viability remained largely unaffected whilst still producing a cellular response to chemical exposure in spermatogonia, spermatocytes and spermatids respectively. The treated and untreated germ cells were used in the Comet assay and qPCR assay.

Determination of Cytotoxicity with Cell Counting Kit-8 (CCK-8) Assay

Cell viability was determined using a modified cell counting kit-8 (CCK-8) Cytotoxicity Assay (Sigma-Aldrich, UK). Spermatogonia, spermatocytes and spermatids were plated in a 96-well plate at a concentration of 5000 cells per well. Ten µl of different concentrations of ENU, 6-MP and MMS (0, 0.05, 0.5 and 1 mM) was added into the culture media in the plate. Cells were pre-incubated for 24 h in a humidified incubator at 37 °C, 5% CO2. Ten µl of CCK-8 solution was added to each well of the plate, followed by incubation at 37 °C for 4 h. Absorbance was measured at a wavelength of 450 nm using a Microplate reader MRX II (Dynex Technologies, Chantilly, USA).

The Comet Assay

The Comet assay was used for assessing the DNA damage in spermatogonia, spermatocyte and spermatid cells after ENU, 6-MP and MMS treatment. Approximately 2 x 10^5 germ cells were suspended in Eppendorf® tubes and incubated with ENU, 6-MP and MMS at final concentrations of 0, 0.05, 0.5, and 1 mM, and grown in 5% CO_2 and 95% air at 37°C. Cell debris was removed and cells remaining in the plates from each treatment were harvested by centrifugation and then used for the examination of DNA damage using the Comet assay as described previously [Habas et al. 2016]. Olive tail moment (OTM) and % tail DNA were measured, calculated, quantified and expressed (fold of control) in mean ± S.D (n=3)

for isolated germ cells using the (Comet 6.0; Andor Technology, formerly Kinetic Imaging) software image analysis system, Belfast, UK.

Quantitative Real-Time PCR Assay

Real-time PCR of *p53* and *bcl-2* genes in cells of spermatogonia, spermatocytes and spermatids after ENU, 6-MP and MMS treatment respectively were examined. 2×10^5 cells/well in 6-well plates were incubated with 0, 0.05, 0.5 and 1 mM of ENU, 6-MP and MMS for 24 h. The cells from each treatment were harvested by centrifugation and the total RNA was extracted using TRIzol® following the manufacturer's (Invitrogen) manual and RNA quantity and quality were checked by OD260/280 measurements. To remove any genomic DNA, the RNA was treated with DNase I (Sigma–Aldrich) according to the manufacturer's instructions. Random hexamer primed reverse transcription reactions were performed for 400 ng of total RNA in a 20 µl setup using ImProm-II™ Reverse Transcription System reaction following the manufacturer's instructions (Promega). The synthesised cDNA samples were diluted 1:10 in nuclease free water and stored at −20 °C. Each assay was run on StepOnePlus™ real-time PCR instrument (Applied Biosystems) in triplicate and expression fold-changes were derived using the comparative CT method.

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.

RESULTS

The cytotoxicity assay using the CCK-8 kit was performed to directly determine the effect of ENU, 6-MP and MMS on cellular viability of spermatogonia, spermatocytes and spermatids respectively under our laboratory conditions. Spermatogonia, spermatocytes and spermatids respectively were either treated with different concentrations of ENU, 6-MP and MMS (0, 0.05, 0.5 and 1 mM) or left untreated and considered as control. The results showed that ENU, 6-MP and MMS at the concentrations 0, 0.05, 0.5 and 1 mM had no significant effect on cell viability (Data not shown). In addition the viabilities of the freshly isolated spermatogonia, spermatocytes and spermatids were routinely >98%, as evidenced by trypan blue exclusion of these cells (Phillips, 1973). The germ cells were cultured overnight at 37°C. The following day, viability was re-checked and the cells treated with the mutagens ENU, 6-MP and MMS. Viabilities were checked again and were found to be routinely >90% for cells that had been exposed to ENU, 6-MP and MMS. They were then used immediately for qPCR and the Comet assay. The different types of cells varied in their ability to respond to the three chemicals. Spermatogonia were most sensitive to ENU, with both DNA damage and gene expression changes (*p53* upregulation and *bcl-2* downregulation) significant at 0.05 mM (Table I, Figs 1 and 2 and Figs 3 A and B). It was also shown that spermatogonia had a significant effect in both assays, when treated with 0.5 and 1 mM 6-MP and MMS. This reflects the amount of actively dividing cells in these cell types, suggesting a possible mechanism for the differential sensitivity. There were concentration-dependent increases in both assays for 6-MP. The concentration that induced a statistically significant increase in genetic damage was 0.[Anderson, 1981 #209]5 mM 6-MP for DNA damage, and 0.5 mM 6-MP for upregulation of *p53* and downregulation of *bcl-2*

mRNA expression (Table I, Figs 1 and 2 and Figs 4 A and B). These results illustrate that there is good agreement with data showing that *in vivo*, 6-MP is a potent compound for inducing DNA damage in spermatocytes.

Spermatids were the most sensitive cell type to MMS, with both DNA damage and gene expression changes (*p53* upregulation and *bcl-2* downregulation) significant at 0.05 mM, 0.5 mM and 1 mM (Table I, Figs 1 and 2 and Figs 5 A and B). Three compounds were significantly genotoxic in cultured male germ cells.

DISCUSSION

Mutant frequencies in male rat germ cells were determined after exposure to ENU, MNU, 6MP, 5BrdU, MMS and EMS and thus have been reported to cause specific DNA damage in isolated germ cells from adult rat testis [Ehling et al. 1978; Anderson et al 1981; Anderson et al. 1997; Russell et al. 2007; Levkoff et al. 2008; Kanemitsu et al. 2009; Habas et al. 2016]. To examine species differences in the DNA damage between rats and mice three male germ cell mutagens (ENU, 6-MP and MMS) were selected for testing. Numerous strategies for the evaluation of reproductive genotoxicity of chemical compounds have been proposed. In the present study, we developed experimental *in vitro* assays to test the effect of potential toxicants on male mouse germ cells *in vitro* using recently developed methods for isolation and culture of adult rats. In fact, there is no recently reliable cell culture system allowing for spermatogenic differentiation *in vitro*, and most biological studies of spermatogenic cells require tissue harvest from animal models like the mouse and rat [Bryant et al. 2013]. Although a reproductive toxicity study has shown that spermatogenesis is a very complex process of which only some stages can be reconstructed *in vitro*. Our study of an *in vitro* model of spermatogenic cells could

make it easier to understand the mechanisms underlying spermatogenic cell differentiation with potential for extrapolation to humans in which experimental approaches are not possible. It could also be used cells isolated from human cadavers. In addition isolated germ cells could greatly improve and make the actual procedures of assisted reproductive technology more efficient and help to develop alternative infertility treatments. The differences in composition and metabolism between different types of male germ cells led to differing susceptibilities to genotoxicity and mutation induction. This is an important toxicological consideration, especially if any *in vitro* study is to be undertaken. This also can provide vital evidence about the possible mechanisms included in genotoxicity and therefore increases the significance of the findings [Habas et al., 2016].

In this present study, we examined DNA-strand breakage induced by ENU, 6-MP and MMS and regulation of the DNA damage response in mouse testicular cells. These three chemicals are well established as reproductive genotoxins and showed clear cell-type specificity *in vivo* and *in vitro* on isolated germ cells from rat testis [Habas et al, 2016]. Genotoxicity assessment in isolated male mice germ cells after exposure to ENU, 6-MP and MMS was conducted using the alkali version of the Comet assay to detect DNA strand breaks and the level of expression of regulated genes *p53* and *bcl-2* for apoptosis were quantified by real-time reverse transcription-PCR (RT-PCR).

Major differences were observed between different cellular phases of mouse spermatogenesis. Spermatogonia were the most sensitive to ENU; spermatocytes were most sensitive to 6-MP while spermatids were the most sensitive cell type to MMS. DNA lesions induced by exposure to 6-MP was higher in rat spermatocytes compared to mouse spermatocytes, indicating major differences in sensitivity

between the two species. The Comet assay results significantly indicate that these results match the positive results found *in vivo* and the cell-type specificity of ENU, 6-MP and, MMS found *in vivo* was the same as that which we found with our *in vitro* system in rat. We have previously reported that treatment of the isolated germ cells from adult rat testis with ENU produced the greatest concentration-related increase in DNA damage in spermatogonia [Habas et al 2016]. There is no prior information to show that ENU inhibited DNA gene expression in spermatogonial cells more than spermatocytes and spermatids and so was investigated in the present study. We also confirmed that ENU upregulation of *p53* and downregulation of *bcl-2* mRNA expression was greatest in spermatogonia. The ENU is extremely mutagenic, and induces apoptosis after S-phase accumulation of *p53* in response to DNA damage [Katayama et al. 2002]. The level of *p53* mRNA was increased similarly, and also in a concentration-dependent manner in the spermatogonia with the lowest concentration of ENU. This was not detected in other cell types, namely spermatocytes or spermatids; mRNA expression of *bcl-2* was also decreased in spermatogonia more than in spermatocytes and spermatids when treated with the lowest concentration of ENU. This result confirms that of a previous study *in vivo*, in which differentiating spermatogonial cells were observed to be the most sensitive testicular cells to ENU damage, due to their higher mitotic rate compared to other spermatogenic cells [Russell et al. 2007]. The data presented here show that the expression level of p53 is high in spermatogonial cells compared to levels in spermatocytes and spermatids; a potential consequence of actively dividing spermatogonia which are the most susceptible male germ cell. Spermatids also show some increased expression of *p53*, yet are non-cycling, differentiating cells with limited DNA repair capacity. Thus it may be that although *p53* is a

multifunctional protein, the principal role of the increased expression observed is to trigger apoptosis. The decreased expression of *bcl-2* and induction of apoptosis were both shown in parallel to the increase in *p53*, which in turn also reflected the induction of DNA damage shown in the Comet assay.

A similar trend was observed in the spermatocytes, which illustrated a highly significant increase in DNA damage and *p53* expression was significantly increased after 6-MP treatment, whereas *bcl-2* was reduced greatly after exposure to 6-MP. In animal models, 6-MP has been shown to cause chromosomal damage and aberrations in the spermatocytes [Russell and Hunsicker 1987]. The cytogenetic study of spermatocyte cells at diakinesis showed a significant increase in isochromatid and chromatid deletions on days 14 and 15 after treatment with 6-MP [Generoso et al. 1975]. This suggested that the cell exposure during this time may have been in early meiosis preleptotene spermatocytes stage. 6-MP has been found to act mainly on rapidly dividing cells (i.e. in the later stages of spermatogenesis) [Maltaris et al. 2006]. It also has shown that 6-MP caused chromosomal damage and aberrations in the spermatocytes [Russell and Hunsicker 1987]. Therefore, spermatocytes were highly sensitive to 6-MP. The results of our *in vitro* experiments are in agreement with the results *in vivo* that suggested that 6-MP is a potent compound for inducing DNA damage in spermatocytes [Mosesso and Palitti 1993].

Between rodents, it's well-established that mice are the most commonly used animal model for studying human disease germ cell differentiation, sex determination and genetics. Study by Encinas et al (2012) who compared the rat and mouse germ cell development, particularly on some germ cells markers including germ cell nuclear antigen 1 (GCNA1), OCT4, mouse vasa homologue (MVH) and specific surface embryonic antigen 1 (SSEA1) were immunolabeled at different phases of embryonic

and postnatal development (Encinas et al., 2012), comparable to our study. Therefore, the level of DNA damage is dependent on both the gentoxicant and the type of germ cells. Also these differential responses to induced DNA damage may contribute to the difference in susceptibility to these three compounds in these three types of cells and species. In this present study, we found that the DNA lesion induced by exposure to 6-MP was higher in rat spermatocytes compared to mouse spermatocytes, indicating major difference in sensitivity between two species. Given the results of the present study in mouse, we note that 6-MP at 0.05 mM produced significantly induced DNA damage in rat, but not in mice. One possible explanation for the difference in sensitivity seen between mouse and rat could be a lower spermatogenic recovery in rat but also the rat may be more sensitive to testicular damage after treatment with this compound than mice. So, we suggest that the rat is also a very important model for the investigation of the mechanisms and susceptibility of germ cells to genotoxicity. After MMS treatment, the sensitive cellular stage for induction of sperm morphological abnormalities was judged to be late spermatids [Kuriyama et al. 2005]. These results also indicated that all three compounds induced DNA damage in isolated germ cells in mouse as well as shown earlier in rat so strengthening the rodent data base. A combination of the Comet assay and qPCR can offer more information relating to biological function in sperm perhaps leading to male infertility.

CONCLUSION

The level of gene expression in response to DNA damage in mouse is dependent on the gentoxicant and the type of germ cells *in vitro*, following concentrations known to be related to testicular and reproductive toxicity *in vivo*. These results indicate that Staput isolated mouse testicular germ cells provide a suitable model *in vitro* to study

DNA damage and regulation of either pro-apoptotic or anti-apoptotic gene expression in different phases of the spermatogenic cycle. The high correlation between the *in vivo* data and the present results indicate this approach could have the possibility to be scaled up into a screen for genetic effects on reproductive cells *in vitro*

STATEMENT OF AUTHOR CONTRIBUTIONS

Dr Khaled Habas carried out the laboratory work; Prof Diana Anderson designed the work for this study, and reviewed all the data. Dr Martin. H. Brinkworth and Prof Diana Anderson jointly supervised the PhD thesis.

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CONFLICT OF INTEREST STATEMENT

The corresponding author confirms that the authors have no conflicts of interest with regard to the funding of this research.

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Figure Legends

Figure 1: Induced DNA damage in germ cells after treatment with ENU, 6-MP and MMS at different concentrations 0, 0.05, 0.5 and 1 mM for 1 h. OTM was used for DNA damage quantification. (*P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group).

Figure 2: Induced DNA damage in germ cells after treatment with ENU, 6-MP and MMS at different concentrations 0, 0.05, 0.5 and 1 mM for 1 h. %tail DNA was used for DNA damage quantification. (*P <0.05, **P <0.01, *** P <0.001 when compared with the respective control group).

Figure 3 A and B: Concentration-dependent effects of ENU on *p53* and *bcl-2* mRNA expression levels in spermatogonia, spermatocytes and spermatids, treated with different concentrations of ENU 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 A and B: Concentration-dependent effects of 6-MP on *p53* and *bcl-2* mRNA expression levels in spermatogonia, spermatocytes and spermatids, treated with different concentrations of 6-MP 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 A and B: Concentration-dependent effects of MMS on *p53* and *bcl-2* mRNA expression levels in spermatogonia, spermatocytes and spermatids, treated with different concentrations of MMS 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.

Table I. Individual data for the effects of ENU, 6-MP and MMS on isolated germ cells measured using the comet parameters: OTM and % tail DNA. Data shown represent group values (mean ± SEM) of three experiments (100 cells scored per experiment)

Germ cells	Olive tail moment	(%)Tail DNA
Spermatogonia		
Control	1.23 ± 0.06	7.23 ± 0.33
0.05mM ENU	2.33 ± 0.13 **	8.37 ± 0.21 **
0.5mM ENU	6.21± 0.49 ***	21.29 ± 1.51***
1mM ENU	9.29 ± 0.20***	26.10 ± 0.64***
Spermatocytes		
Control	0.91 ± 0.08	4.97 ± 0.35
0.05mM ENU	1.35 ± 0.23 ns	7.28 ± 0.65 ns
0.5mM ENU	3.01 ± 0.15 *	12.73 ± 1.15*
1mM ENU	3.78 ± 0.21**	15.45 ± 1.27**
Spermatids		
Control	0.59 ± 0.05	4.50 ± 0.35
0.05mM ENU	0.75 ± 0.09 ns	7.21± 0.69 ns
0.5mM ENU	1.87 ± 0.37 *	9.82 ± 0.92 *
1mM ENU	2.66 ± 0.28**	13.42 ± 0.85 **
Spermatogonia		
Control	0.90 ± 0.07	3.93 ± 0.27
0.05mM 6-MP	1.35 ± 0.29 ns	5.26 ± 0.96 ns
0.5mM 6-MP	1.77 ± 0.15*	7.57 ± 0.54*
1mM 6-MP	2.74 ± 0.14**	10.67± 0.97**
Spermatocytes		
Control	0.86 ± 0.08	4.04 ± 0.58
0.05mM 6-MP	1.66 ± 0.27 ns	7.15 ± 0.47ns
0.5mM 6-MP	3.44 ± 0.33**	12.99 ± 1.15**
1mM 6-MP	5.01 ± 0.18***	17.43 ± 0.90***
Spermatids		
Control	0.85 ± 0.09	4.02 ± 0.60
0.05mM 6-MP	1.60 ± 0.28ns	6.10 ± 0.99ns
0.5mM 6-MP	2.08 ± 0.33*	7.87 ± 1.06*
1mM 6-MP	3.00 ± 0.46**	11.94 ± 1.73**
Spermatogonia		
Control	0.99 ± 0.04	3.96 ± 0.24
0.05mM MMS	2.03 ± 0.44 ns	7.64 ± 0.92ns
0.5mM MMS	4.48 ± 0.63*	10.69 ± 1.06*
1mM MMS	5.24 ± 0.79**	17.63 ± 1.92**
Spermatocytes		
Control	1.07 ± 0.12	4.59 ± 0.94
0.05mM MMS	1.90 ± 0.10 *	10.75 ± 0.94*
0.5mM MMS	4.85 ± 0.68**	11.93 ± 1.75**
1mM MMS	6.48 ± 0.85**	18.82 ± 1.16**
Spermatids		
Control	1.18 ± 0.37	4.91 ± 0.62
0.05mM MMS	3.31 ± 0.30 **	13.01± 1.00 **
0.5mM MMS	8.84 ± 1.08***	26.78 ± 0.86***
1mM MMS	12.00 ± 1.05***	37.30 ± 1.41***

Figure 1

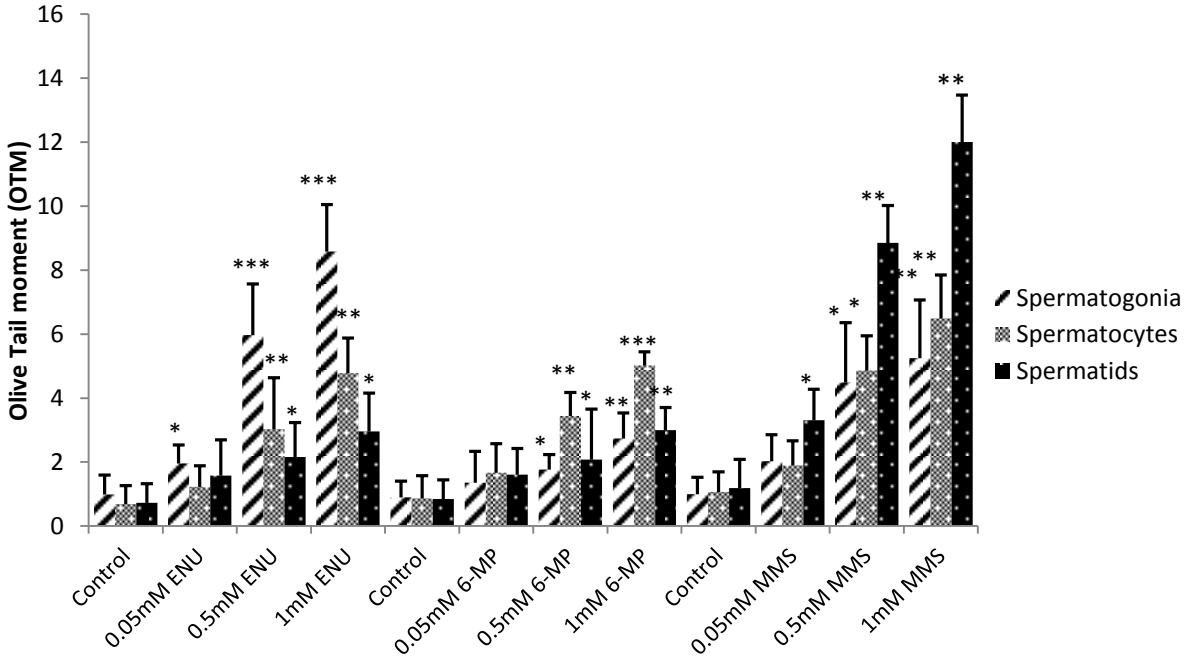

Olive Tail moment (OTM)
16
14
12
10
8
6
4
2
0
Control
0.05mM ENU
0.5mM ENU
1mM ENU
Control
0.05mM 6-MP
0.5mM 6-MP
1mM 6-MP
Control
0.05mM MMS
0.5mM MMS
1mM MMS
Spermatogonia
Spermatocytes
Spermatids

Figure 2

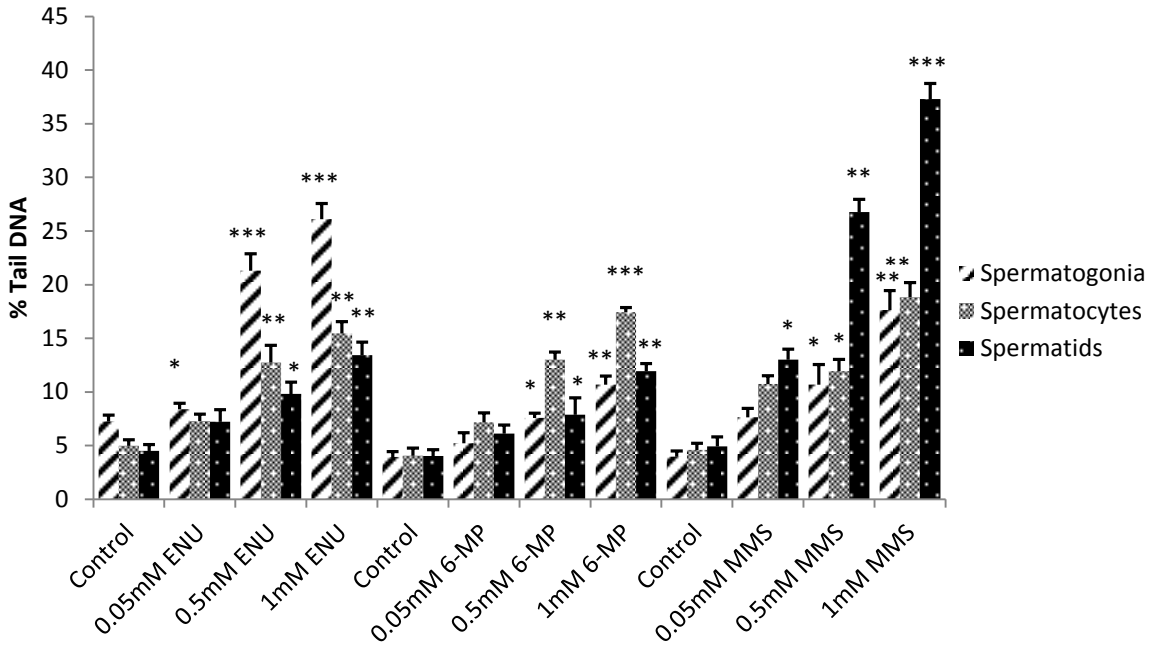
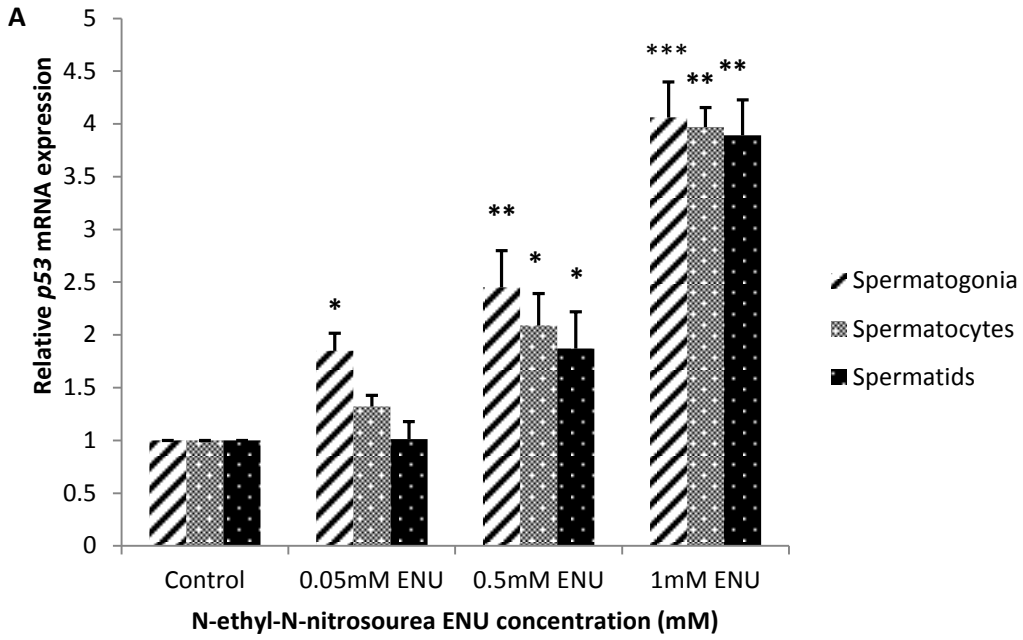

% Tail DNA
45
40
35
30
25
20
15
10
5
0
Control
0.05mM ENU
0.5mM ENU
1mM ENU
Control
0.05mM 6-MP
0.5mM 6-MP
1mM 6-MP
Control
0.05mM MMS
0.5mM MMS
1mM MMS
Spermatogonia
Spermatocytes
Spermatids

Figure 3 A and B



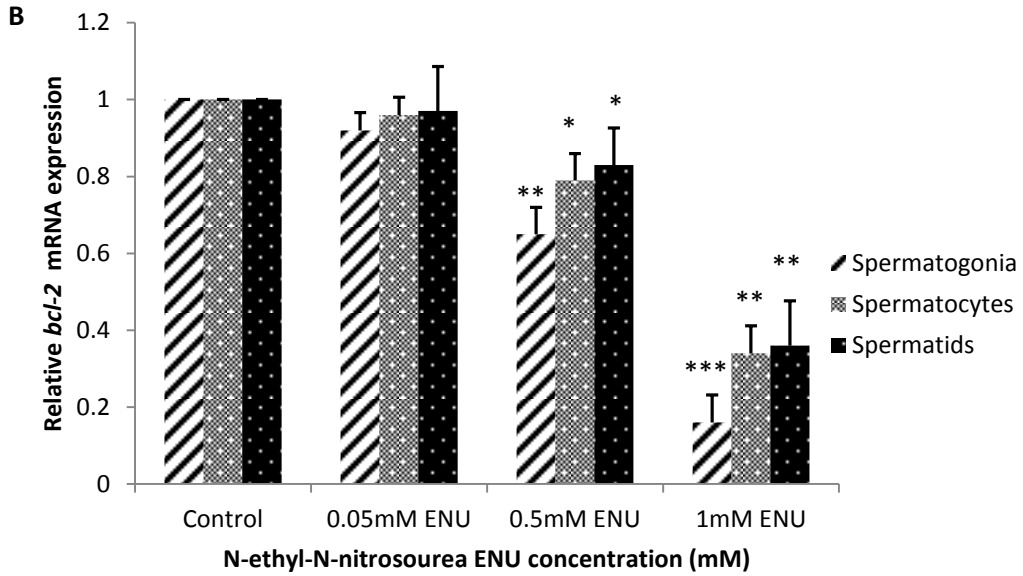
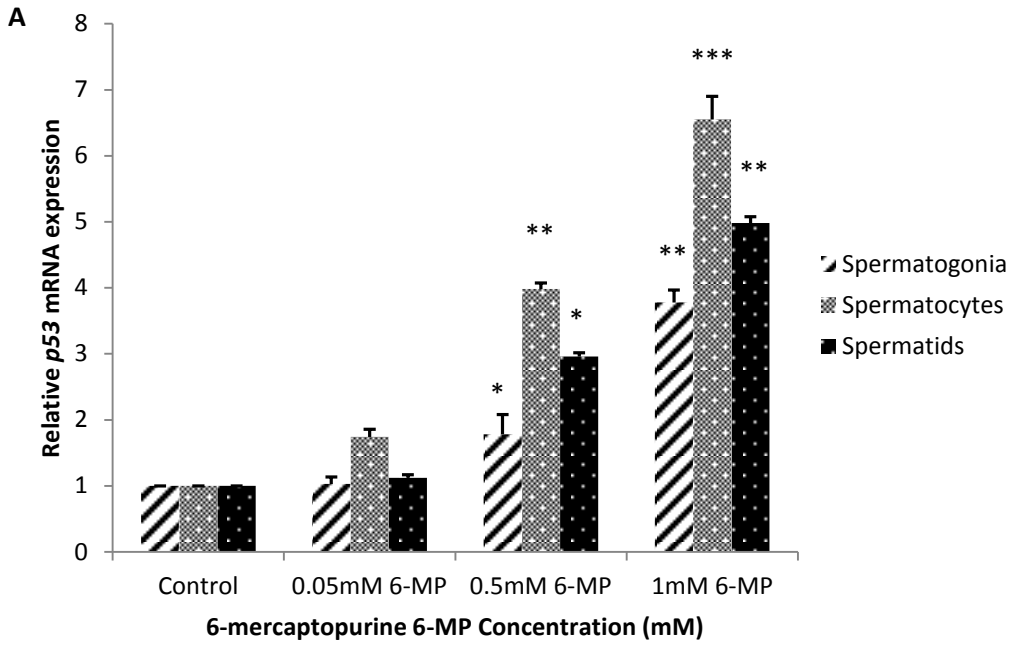


Figure 4 A and B



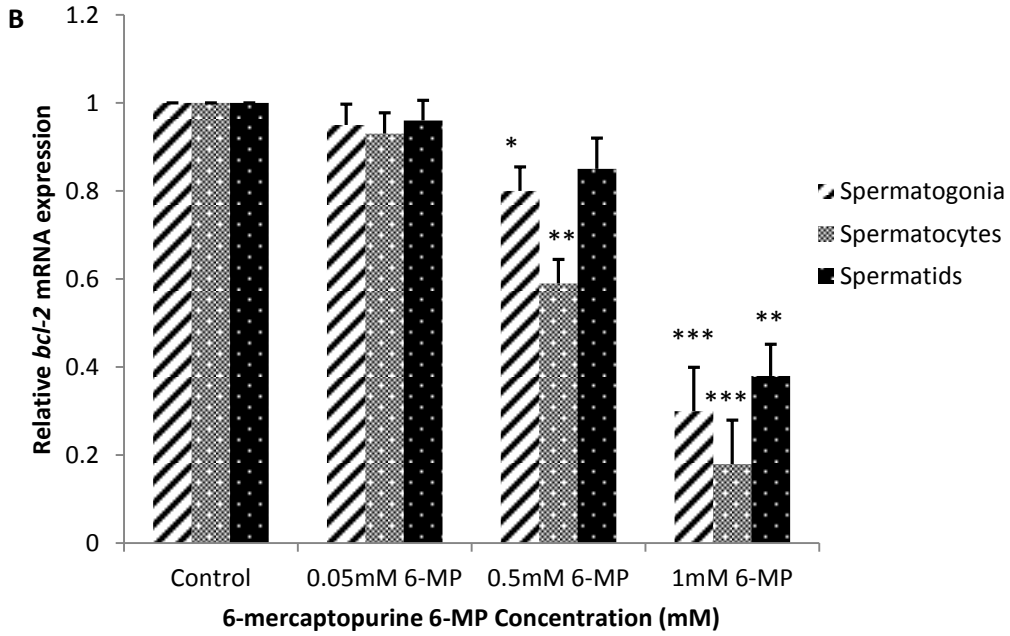


Figure 5 A and B