Responses to Genotoxicity in Mouse Testicular Germ Cells and Epididymal Spermatozoa are Affected by Increased Age.

Running title: Genotoxic effects on germ cells in young and aged male mice.

J.D. Taylor[2], A. Baumgartner[2], T.E. Schmid[3] and M.H. Brinkworth[1]*

[1]School of Chemistry & Biosciences, University of Bradford, Bradford, UK
[2]Dept. Biomedical Sciences, School of Health Sciences, York St John University, York, UK.
[3]Department of Radiotherapy, Klinikum rechts der Isar, Technical University of Munich, Germany

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* To whom correspondence should be addressed:
Tel.: +44 (0)1274 233584
Fax: +44 (0)1274 309742
Email: M.H.Brinkworth@Bradford.ac.uk

Abstract

The increased number of cell divisions undergone by spermatogonia of older fathers cannot fully account for the observed increase in germline genetic damage. Studies have shown that the mechanisms induced in germ cells in response to oxidative damage varies with age, that DNA repair efficiency declines, and both sperm DNA damage and spontaneous mutations increase. However, it is not known whether the altered response with age is a cause, or consequence, of an age-associated change in cell susceptibility to genetic damage.

Following a single 150 mg/kg dose of cyclophosphamide (CP), young (8-weeks old) and aged (17-month old) male mice were examined 24h later for induced genetic damage in epididymal spermatozoa using the alkaline comet and sperm chromatin stability assays. Apoptosis among testicular cells was examined on tissue cross-sections using the TUNEL assay.

Sperm showed no significant increase in DNA strand breaks with age (detected by the comet assay) and no change in sperm chromatin stability (detected by the SCSA assay). Following CP treatment, there was no effect on DNA-strand breakage but sperm chromatin instability was significantly higher. Furthermore, it was also significantly elevated in old treated, compared with young treated, animals suggesting that increased age affects the sensitivity of epididymal sperm to chromatin damage.

There was no difference in apoptosis in testicular germ cells from either young or old control animals, while CP administration resulted in a significant increase in apoptosis among young animals but not old animals. Following genotoxin exposure, an increase in chromatin instability in the spermatozoa of old animals and a decrease in the ability of their testicular germ cells undergo apoptosis suggests an age-related decrease in genome protection mechanisms. Since those germ cells are only transiently present in the testis, it is likely that this age-related deterioration originates in the spermatogonial stem cells. The findings are also evidence that the safety evaluation of reproductive genotoxins should consider young and old individuals separately.

Introduction

Paternal ageing results in an increase in germ-line genetic damage that is known to contribute to a number of paternally-mediated genetic diseases (Chianese et al., 2014; Conti and Eisenberg, 2016; Gunes et al., 2016; Herati et al., 2017; Katz-Jaffe et al., 2013; Lazarou and Morgentaler, 2008; Paul and Robaire, 2013; Zhang et al., 2006). In somatic cells, it has been shown that mutation rates are significantly higher than in germ cells (Milholland et al., 2017), indicating that the germline genome is better protected from genetic damage. However, it has also been reported that the number of *de novo* mutations acquired in the male germ-line doubles every 16.5 years (Kong et al., 2012). Males maintain fertility throughout life (in the absence of other pathology) so developing germ cells necessarily derive from a pool of spermatogonia present from birth. As the males age, they produce germ cells that have undergone a higher number of cell divisions, and cell division is principally when mutations are induced. Nevertheless, it was demonstrated by Crow (1997) on mathematical grounds, that the increased number of cell divisions associated with spermatogenesis in older fathers, could not solely account for the increased number of mutations observed in their offspring (Crow, 1997).

A recent study has shown that the response of aged catalase-deficient knockout mice to induced oxidative stress is different from that of young animals (Selvaratnam and Robaire, 2016) and the authors suggested that old, but not young animals have mechanisms to partially compensate for the lack of catalase activity. Ageing has also been shown to cause a reduction in the efficiency of germline DNA repair (Yatsenko and Turek, 2018) and an increase in sperm DNA damage (Kaarouch et al., 2018) and the number of spontaneous mutations (Maher et al., 2018).

It has long been known that therapeutic use of the potent oxazaphosphorine alkylating agent Cyclophosphamide adversely affects semen parameters (Anderson et al., 1995). For the treatment of cancer (Veal et al., 2016), arthritis (Tiseo et al., 2016) and kidney disease (Gajjar et al., 2015), CP has been shown to increase sperm aneuploidy (Martinez et al., 2017), germ-cell genetic damage (Liu et al., 2014) and germ-cell loss (Smart et al., 2018). Further, when used in childhood chemotherapy, CP has also been shown to cause dose-dependent long-term testicular damage, detectable when

childhood (pre-pubertal) survivors of leukaemia reach adulthood (Green et al., 2014; Servitzoglou et al., 2015).

CP is regarded as a model genotoxin, used to induce predictable patterns of genetic damage and hence provide the opportunity to examine the effects of exposing specific germ-cell types to a particular genotoxin (Grenier et al., 2012; Szikriszt et al., 2016). CP undergoes activation principally through the cytochrome P450 enzyme CYP2B6, to yield the toxic metabolite acrolein (reviewed in: Stevens, 2008) and the alkylating agent phosphoramide (Brock, 1996; Connors et al., 1974; Sladek, 1988). *In vivo*, these metabolites induce a variety of types of DNA damage, including adduct formation (Brock, 1996; Wheeler, 1962) and chromosomal rearrangements (Esposito et al., 1989; Martin et al., 1985). CP has been used to model the mechanisms of genotoxicity in both humans and animals (Aguilar-Mahecha et al., 2001, 2005) and the effects of exposure to CP in the germline of young adults is well characterised (Aguilar-Mahecha et al., 2005; Codrington et al., 2004; Connors et al., 1974; Fairley et al., 1972; Gligor and Gligor, 1995; Harrouk et al., 2000; Kohler et al., 1991; Mohn and Ellenberger, 1976; Trasler et al., 1988; Trasler and Robaire, 1988).

Accordingly, the present study utilised CP as a model genotoxin to determine whether the responses of epididymal sperm and germ cells from old mice differ from those of young mice in terms of DNA strand breaks in spermatozoa and the induction of apoptosis in testicular germ cells *in vivo*. This has toxicological significance as it will help clarify whether there is a need for age-differentiated risk estimates for exposure to genotoxins.

Materials and Methods

Study design, animal treatment and sample collection

Young (2-month old, about 10% of the average mouse lifespan) and aged (17-month old, about 85% of average lifespan) male (102/El x C3H/El) F_1 mice were randomly assigned to treatment or control groups (n=10 per group). Animals were weighed and treated by single, intraperitoneal injection of vehicle (0.9% w/v NaCl) or 150 mg/kg CP and housed under a 12 hour light:dark cycle at the animal house of the Klinikum rechts der Isar, Technical University of Munich, Germany. The District Government of Upper

Bavaria approved all animal experiments. Food and water were provided *ad libitum*. Animals were sacrificed 24 hours post-treatment and both the testes and cauda epididymides removed. Epididymal sperm were collected by incising the caudae several times and incubating them in 300µl foetal calf serum (FCS) (Gibco, UK) at 30°C for 30 min. FCS was used to prevent osmotic shock (Lowe et al., 1996; Schmid et al., 1999). After incubation, epididymal tissue was removed and the sperm suspension frozen in liquid nitrogen without additional media or cryoprotectants. Samples were stored at -80°C until use. Testes were weighed and then fixed in Bouin's fixative (180mM saturated aqueous picric acid; 1M formaldehyde; 20mM glacial acetic acid (all from Fluka, Germany) for 24 hours. The fixative was removed and the testes rinsed extensively in lithium carbonate saturated 70% ethanol (EtOH) to remove picric acid staining. Decolourised testes were embedded using a Shandon automatic tissue processor.

Processed tissue was paraffin embedded and 4µm sections mounted on 1% poly-L-lysine (Sigma, UK) coated, glass slides.

Sperm counts

Sperm counts were performed according to standard protocols adapted for mouse from the World Health Organisation protocols for human sperm counting (World Health Organisation, 2010). The final dilution of the sample was dependent upon the number of sperm visible per field of view on an initial wet preparation (see: World Health Organisation, 2010). After dilution in counting diluent (0.6M $NaHCO_3$ in water with 1% v/v of 38% w/v formaldehyde), sperm were loaded onto an improved Neubauer haemocytometer and incubated at room temperature in a humidified box for ten minutes, allowing the sperm to settle onto the grid of the chamber. Approximately two hundred sperm per sample were counted in duplicate (World Health Organisation, 2010).

Alkaline sperm Comet assay

DNA strand breakage was measured with the alkaline Comet assay. Sperm were defrosted and briefly centrifuged. The supernatant was removed and the pellet re-suspended in 100 µl 1% w/v low melting point (LMP) agarose (Sigma, UK) made in phosphate buffered saline (PBS). The agarose-sperm mixture was applied to agarose-

coated glass slides, cover-slipped and allowed to solidify. A top layer of 0.5% LMP agarose was added, the slide was again covered and the agarose allowed to set. Slides were incubated for 1 hour in lysis buffer at pH 10 (2.5 M NaCl; 100 mM EDTA; 10 mM Trizma base; 40 mM dithiothreitol; 10% v/v dimethyl sulphoxide; 1% v/v Triton X-100 (all Sigma, UK) at 37°C. Proteinase-K was added to a final concentration of 0.05 mg/ml (Promega, UK) and the slides incubated for a further 3 hours at 37°C. Slides were removed from lysis buffer and placed in the electrophoresis tank containing fresh electrophoresis buffer (300 mM NaOH; 1mM EDTA, pH >13) at 4°C. The DNA was allowed to unwind for 20 minutes before electrophoresis in the same buffer for 20 minutes at 0.75V/cm. Slides were neutralised with 25 mM Tris-HCl (pH 7) and stained with ethidium bromide (Sigma, UK). The percentage head DNA and tail moment of fifty cells per slide on two slides were scored using the Kinetic Imaging (UK) Komet-4® software.

Sperm Chromatin Stability Assay (SCSA)

The Sperm Chromatin Stability Assay (SCSA) was carried out following the procedure of Evenson & Jost (Evenson and Jost, 1994; Evenson, 2013) at the Clinic of Veterinary Medicine of the Ludwig-Maximilian-University, Munich, Germany.

Frozen sperm was thawed and diluted in TNE buffer (150 mM NaCl; 10 mM Tris and 1.0 mM EDTA pH 7.4) to a concentration of 1-2x10^6 sperm/ml. Four hundred microlitres of acidic detergent (80 mM HCl; 150 mM NaCl; 0.1% v/v Triton X-100, pH 1.2) was added to 200µl of the diluted sperm sample. After 30 seconds, sperm were stained by addition of 1.2ml acridine orange solution (0.06% w/v acridine orange in buffer [37mM citric acid; 126mM Na_2HPO_4; 11mM EDTA; 15mM NaCl, pH 6.0]) and stained for three minutes. All steps were performed on ice. After staining, 5000 sperm per sample were analysed by a FACStar Plus flow cytometer with a 200mW argon-ion laser. When excited by a blue light source, acridine orange intercalates with double stranded DNA exhibits green fluorescence (530±30nm) and when associated with single strand DNA it exhibits red fluorescence (>630nm). Data were expressed by the function alpha-t (αT), which is the ratio of red to red+green fluorescence. This represents the total amount of denatured (single-stranded) DNA over the total cellular (single and double-stranded) DNA. Results are expressed as the percentage of cells that fall outside the main population (%DFI) (Evenson, 2013; Evenson et al., 1991).

Terminal Deoxyuridine Nick End Labelling (TUNEL)

Sections were de-paraffinised and re-hydrated though a 100% n-butyl acetate and ethanol (EtOH) series (2 x n-butyl acetate → 2 x 100% EtOH → 2 x 90% EtOH → 2 x 70% EtOH → deionised [d]H_2O) for 5 min each. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 15 min at room temperature. Sections were rinsed with terminal deoxyuridine transferase (TdT) buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride; Sigma, UK). 10 µM biotin-16-deoxyuridine triphosphate (Sigma, UK) and 0.3 U/µl TdT (Promega, UK) in TdT buffer were added to cover the sections, which were incubated in a humidified box at 37°C for 60 min. The labelling reaction was terminated by washing slides in Tris buffered saline (TBS) (50 mM Tris; 375 mM NaCl, pH 7.6; Sigma, UK) for 15 min. The sections were rinsed with dH_2O and the biotin label visualised using a 1:50 dilution of Extravidin-peroxidase (Sigma, UK) in TBS with 0.1% w/v bovine serum albumin (Sigma, UK) and incubated at 37°C for 30 min. Slides were again washed in TBS and incubated with diaminobenzidine (DAB) (Sigma, UK) according to the manufacturer's guidelines. Slides were finally rinsed in water, counterstained for 10 seconds in Mayer's haemalaum (Sigma, UK), dehydrated though an ethanol series and mounted with Histomount® (Vector, UK) (Gavrieli et al., 1992).

Slides were coded and randomised prior to evaluation using a Nikon Eclipse 80*i* microscope at 400x magnification to allow examination of individual tubules. Tubules were scored if the tubule cross-section was intact, rounded and contained within a single field of view, with no obvious signs of physical damage or non-specific staining. Per tubule, the numbers of brown-stained, TUNEL-positive apoptotic cells was recorded. Fifty tubules were scored per animal, but expression of the number of apoptotic cells per tubule cross-section is only valid if there are no significant age- or treatment-associated alterations in the dimensions of the tubules. Therefore, the diameter of twenty rounded tubule cross-sections was measured per animal for each group (Brinkworth and Nieschlag, 2000).

Statistical analysis

Datapoint distribution was assessed for univariate normality using Q-Q analysis. Data were analysed for statistical differences using two-way ANOVA, with Bonferroni post-hoc testing to compare the effects of treatment, age and their interactions (GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA,

www.graphpad.com). For all figures, n=10 per group and data are means ± standard deviation.

Results

Animal and organ data (Table I)

Gross animal weight, gross testes weight, testes weight as percentage bodyweight and sperm count, were not significantly different as a result of either age or treatment. No gross alteration was observed in the seminiferous epithelium, which appeared quantitatively normal at the tubular level, seminiferous tubule diameters and Sertoli cell numbers were consistent among all animals, irrespective of age or treatment.

Alkaline Comet assay (Figure 1)

There was no effect of CP treatment on comet tail moment in either young (8.10 ± 2.75 vs. 10.17 ± 2.53) or old (7.04 ± 2.31 vs. 10.58 ± 4.46) animals, when compared with their respective controls.

SCSA (Figure 2)

The sperm of young treated animals (7.13 ± 2.67) showed a 2.23-fold increase in %DFI vs. young controls (3.20 ± 0.77) ($p<0.01$); similarly, %DFI in sperm of old treated (11.95 ± 3.16) vs. old control (4.92 ± 2.00) animals was increased 2.43-fold ($p<0.001$). There was also a small but significant (0.68-fold) increase in sperm DFI among young treated (7.13 ± 2.67) vs. old treated (11.95 ± 3.16) animals ($p<0.01$). However no statistically significant difference was observed between young control (3.20 ± 0.77) and old control (4.92 ± 2.00) animals ($p>0.05$).

TUNEL (Figure 3)

In the testes, there was a significant 5.17-fold increase in the number of apoptotic testicular germ cells in the seminiferous tubules of young treated (114.2 ± 73.05) vs. young control (22.1 ± 18.11) animals ($p<0.01$). By contrast, there was a significant 5.05-fold reduction in the number of apoptotic cells in the tubules of old treated (22.6 ± 14.93), compared with young treated (114.2 ± 73.05) animals ($p<0.05$).

Although reduced 2.83-fold, the number of apoptotic germ cells in the tubules of old control (7.8 ± 4.44) vs. young control (22.1 ± 18.11) animals was not significantly

different ($p>0.05$) because of an unusually wide range of values in the young control group.

TUNEL tubule cross-sections (Figure 4)

Tubule cross-sections appeared qualitatively normal in all animals. A system negative control (a) to confirm assay specificity showed no evidence of non-specific background staining. 0.9%NaCl treated control animals (b) had clearly identifiable apoptotic cells and no non-specific background staining. However, 150mg/kg CP treated animal tubules (c) all showed significant background staining in all sections, irrespective of age. This was consistent across all treated animals and presumably reflects CP-induced alkylation damage to cellular macromolecules other than DNA. Despite this, apoptotic cells were clearly identifiable compared with the background in all sections.

Discussion

The principal objective of this study was to determine differences in the response of male germ cells to CP in young and old animals. In untreated epididymal sperm from young animals, the levels of DNA damage measured directly by the alkaline Comet assay and indirectly by the SCSA assay, were in line with levels reported for different strains of mice (Perez-Cerezales et al., 2012; Pina-Guzman et al., 2006; Sailer et al., 1995).

We did not observe any effect between young control and old control animals using the SCSA assay to determine acid-denaturation induced, percent DNA fragmentation (%DFI), which provides a measure of chromatin stability (Evenson, 2013, 2016). However, we observed a significant increase in CP treated vs. control animal %DFI irrespective of age. Since this treatment did not yield effects detectable by the alkaline comet assay (DNA strand breakage), it is possible that much of the CP-induced increase in %DFI resulted from alkylation of the protein component of sperm chromatin, rather than the DNA. This has been previously reported for sub-chronic CP administration (Codrington et al., 2007; Vaisheva et al., 2007), but not for a single-dose study, which suggests that CP induced, sperm chromatin-protein damage is a more readily induced phenomenon than sperm DNA damage. This is may result from the highly condensed nature of protaminated sperm chromatin. It is also in agreement

with the lack of any observed DNA strand breakage detected by the Comet assay, which is supported by a previous report in rat sperm (Codrington et al., 2004) where 100mg/kg CP did not produce significant quantities of DNA strand breakage. Nevertheless, the dose of CP used in the present study would be expected to cause some damage to the DNA as it is a powerful inducer of dominant lethal mutations in mouse sperm. CP-induced alkyl adducts are converted into mutations by misrepair (Gillingham and Sauter, 2017; Yauk et al., 2015) and acute exposure of sperm to doses of 100-200 mg/kg has been shown to readily produce dominant lethal effects (Ehling and Neuhauser-Klaus, 1988; Harrouk et al., 2000; Oliveira et al., 2014). Zygotic reprogramming occurs shortly after fertilization and is characterised by sperm chromatin reorganisation and epigenetic reprogramming, including activation of DNA repair mechanisms (Ladstatter and Tachibana-Konwalski, 2016; Zou, 2016), so it is possible that acute CP-induced DNA alkylation in epididymal sperm may subsequently disrupt this reprogramming by producing excessive aberrant DNA strand breaks after fusion of the two pronuclei, resulting in dominant lethal DNA damage.

Chromatin instability was significantly higher in old treated compared with young treated animals, suggesting that increased age affects the sensitivity of epididymal sperm to chromatin damage. It is not possible to say what is causing the increased susceptibility but since it is only the spermatogonial stem cells that age with the individual, it is likely that this is where the defect arises.

In contrast to epididymal sperm, testicular germ cells showed a marked response to CP with a statistically significant, nearly 6-fold increase, in the number of TUNEL positive apoptotic cells in the testes of young treated compared with young control animals. The number of Sertoli cells remained constant between control and treated groups in both old and young animals so normalising the apoptotic germ cells against Sertoli cell count demonstrates that the increased numbers of apoptotic germ cells detected was not an artefact of the loss of other cell types or tubule distortion.

Aguilar-Mahecha et al. (2005) reported that acute, 70mg/kg CP treatment induced G2/M checkpoint arrest and consequently, significant DNA strand-breakage in rat spermatids (Aguilar-Mahecha et al., 2005); similarly, Cai et al. (1997) demonstrated that spermatids could undergo apoptosis within 4–12 hours after treatment with the same dose of CP. It has also been suggested that progression of sperm through the metaphase-I spindle assembly checkpoint (SAC) can be maintained at levels associated with normal sperm production and fertility, despite recombination defects

in up to 50% of spermatocytes (Faisal and Kauppi, 2016). Our observation that it was principally meiotic and pre-meiotic germ cells undergoing apoptosis, potentially reflects the loss of germ cells unable to be repaired at either prophase-I (see: Jan et al., 2018) or metaphase-I (see: Faisal and Kauppi, 2016) checkpoints.

By contrast, the number of apoptotic cells in old treated animals compared with old controls did not show a statistically significant increase. Thus, there is an age-dependent difference in the response of spermatogonia and spermatocytes to CP-induced damage, suggesting that the ability of these germ cells to undergo apoptosis in response to a genotoxic insult is reduced with age. It is unlikely that our observation in old animals is due to the prior loss of susceptible germ cells as a result of life-long *de novo* damage, because the numbers of epididymal sperm, which represent the previous generation of developing germ cells at the time of CP exposure, was not influenced by age. The findings show that spermatogonia, spermatocytes and epididymal sperm in the aged male all show defects that result in an elevation of induced genetic damage persisting in the germline that has the potential to be converted into mutation in the early embryo.

Work on *C. elegans* has demonstrated antagonistic pleiotropy in the germline, whereby maintenance of apoptosis in advanced age causes more rapid gonad degeneration, but this degeneration does not occur in aged worms with reduced levels of apoptosis (de la Guardia et al., 2016). If a similar response occurs in aged mammals, by less-stringent meiotic checkpoint control, this would allow damaged germ cells to progress through meiosis and hence maintain fertility into old age. The lower apoptotic response may confer a more important survival advantage than an error-free paternal genome (Zhao and Epstein, 2008), particularly since DNA repair mechanisms in the zygote repair lesions associated with post-fertilization epigenetic reprogramming (Ladstatter and Tachibana-Konwalski, 2016; Zou, 2016). Presumably such mechanisms could remove a certain amount of additional, pre-existing genetic damage originating during spermatogenesis. Our findings of a decreased apoptotic response of meiotic cells to CP and an increased susceptibility of aged spermatozoa to chromatin damage induced by CP support this. They provide a potential mechanism by which this principle may result in increased genotoxicity and consequently higher mutation rates in offspring via the male germline, following genotoxin exposure of aged individuals.

The extra germ-cell divisions and greater cumulative exposure to environmental and endogenous genotoxins resulting from a longer life span, cannot explain all of the increase in mutation rate in aged males. Our results suggest another mechanism whereby ageing of the stem cells exacerbates the likelihood of acquiring DNA damage and hence mutation via the male germline. In turn, this implies that the safety evaluation of reproductive genotoxins should consider young and old individuals separately.

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