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

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

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25 **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  
30 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  
35 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  
40 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  
45 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  
50 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.

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## 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;  
90 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  
95 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 phosphoramidate (Brock, 1996; Connors et al., 1974; Sladek, 1988). *In vivo*, these  
metabolites induce a variety of types of DNA damage, including adduct formation  
100 (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  
105 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  
110 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

### 115 *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/EI x C3H/EI) F<sub>1</sub> 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  
120 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  
125 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  
130 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.  
135 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  
140 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<sub>3</sub> in water with 1% v/v of 38% w/v formaldehyde), sperm were loaded onto an improved Neubauer  
145 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).

#### 150 *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-

155 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  
160 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)  
165 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<sup>®</sup> software.

#### *Sperm Chromatin Stability Assay (SCSA)*

170 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  $1-2 \times 10^6$  sperm/ml. Four hundred  
175 microlitres of acidic detergent (80 mM HCl; 150 mM NaCl; 0.1% v/v Triton X-100, pH  
1.2) was added to 200 $\mu$ l 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<sub>2</sub>HPO<sub>4</sub>; 11mM EDTA; 15mM NaCl, pH 6.0]) and  
stained for three minutes. All steps were performed on ice. After staining, 5000 sperm  
180 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 $\pm$ 30nm) and when associated with  
single strand DNA it exhibits red fluorescence (>630nm). Data were expressed by the  
function  $\alpha$ -t ( $\alpha$ T), which is the ratio of red to red+green fluorescence. This  
185 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)*

190 Sections were de-paraffinised and re-hydrated through 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<sub>2</sub>O) 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  
195 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)  
200 for 15 min. The sections were rinsed with dH<sub>2</sub>O 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  
205 seconds in Mayer's haemalaum (Sigma, UK), dehydrated through 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 80i 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  
210 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  
215 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  
220 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](http://www.graphpad.com)). For all figures, n=10 per group and data are means  $\pm$  standard deviation.

225

## 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.

230 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)*

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

### *SCSA (Figure 2)*

240 The sperm of young treated animals ( $7.13 \pm 2.67$ ) showed a 2.23-fold increase in %DFI vs. young controls ( $3.20 \pm 0.77$ ) ( $p < 0.01$ ); similarly, %DFI in sperm of old treated ( $11.95 \pm 3.16$ ) vs. old control ( $4.92 \pm 2.00$ ) 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 ( $7.13 \pm 2.67$ ) vs. old treated ( $11.95 \pm 3.16$ ) animals ( $p < 0.01$ ). However no  
245 statistically significant difference was observed between young control ( $3.20 \pm 0.77$ ) and old control ( $4.92 \pm 2.00$ ) animals ( $p > 0.05$ ).

### *TUNEL (Figure 3)*

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

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

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

260 *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  
265 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.

270 **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  
275 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).  
280 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  
285 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,  
290 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  
295 (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  
300 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  
305 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  
310 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  
315 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  
320 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  
325 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-  
330 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  
335 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.

340 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  
345 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  
350 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  
355 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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370

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375

### **References**

380 Aguilar-Mahecha, A., Hales, B.F., Robaire, B., 2001. Acute cyclophosphamide exposure has germ cell specific effects on the expression of stress response genes during rat spermatogenesis. *Mol Reprod Dev* 60, 302-311.

385 Aguilar-Mahecha, A., Hales, B.F., Robaire, B., 2005. Effects of acute and chronic cyclophosphamide treatment on meiotic progression and the induction of DNA double-strand breaks in rat spermatocytes. *Biology of reproduction* 72, 1297-1304.

Anderson, D., Bishop, J.B., Garner, R.C., Ostrosky-Wegman, P., Selby, P.B., 1995. Cyclophosphamide: Review of its mutagenicity for an assessment of potential germ

- cell risks. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 330, 115-181.
- 390 Brinkworth, M.H., Nieschlag, E., 2000. Association of cyclophosphamide-induced male-mediated, foetal abnormalities with reduced paternal germ-cell apoptosis. *Mutat Res* 447, 149-154.
- Brock, N., 1996. The history of the oxazaphosphorine cytostatics. *Cancer* 78, 542-547.
- 395 Cai, L., Hales, B.F., Robaire, B., 1997. Induction of apoptosis in the germ cells of adult male rats after exposure to cyclophosphamide. *Biology of reproduction* 56, 1490-1497.
- Chianese, C., Brilli, S., Krausz, C., 2014. Genomic changes in spermatozoa of the aging male. *Advances in experimental medicine and biology* 791, 13-26.
- Codrington, A.M., Hales, B.F., Robaire, B., 2004. Spermiogenic germ cell phase-specific DNA damage following cyclophosphamide exposure. *J Androl* 25, 354-362.
- 400 Codrington, A.M., Hales, B.F., Robaire, B., 2007. Exposure of male rats to cyclophosphamide alters the chromatin structure and basic proteome in spermatozoa. *Hum Reprod* 22, 1431-1442.
- 405 Connors, T.A., Cox, P.J., Farmer, P.B., Foster, A.B., Jarman, M., 1974. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. *Biochem Pharmacol* 23, 115-129.
- Conti, S.L., Eisenberg, M.L., 2016. Paternal aging and increased risk of congenital disease, psychiatric disorders, and cancer. *Asian journal of andrology* 18, 420-424.
- Crow, J.F., 1997. The high spontaneous mutation rate: is it a health risk? *Proc Natl Acad Sci U S A* 94, 8380-8386.
- 410 de la Guardia, Y., Gilliat, A.F., Hellberg, J., Rennert, P., Cabreiro, F., Gems, D., 2016. Run-on of germline apoptosis promotes gonad senescence in *C. elegans*. *Oncotarget*.
- Ehling, U.H., Neuhauser-Klaus, A., 1988. Induction of specific-locus and dominant-lethal mutations by cyclophosphamide and combined cyclophosphamide-radiation treatment in male mice. *Mutat Res* 199, 21-30.
- 415 Esposito, D., Fassina, G., Szabo, P., De Angelis, P., Rodgers, L., Weksler, M., Siniscalco, M., 1989. Chromosomes of older humans are more prone to aminopterin-induced breakage. *Proc Natl Acad Sci U S A* 86, 1302-1306.
- Evenson, D., Jost, L., 1994. Sperm chromatin structure assay: DNA denaturability. *Methods in cell biology* 42 Pt B, 159-176.
- 420 Evenson, D.P., 2013. Sperm chromatin structure assay (SCSA(R)). *Methods Mol Biol* 927, 147-164.
- Evenson, D.P., 2016. The Sperm Chromatin Structure Assay (SCSA((R))) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim Reprod Sci* 169, 56-75.

- 425 Evenson, D.P., Jost, L.K., Baer, R.K., Turner, T.W., Schrader, S.M., 1991. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol* 5, 115-125.
- Fairley, K.F., Barrie, J., Johnson, W., 1972. Sterility and Testicular Atrophy Related to Cyclophosphamide Therapy. *The Lancet* 299, 568-569.
- 430 Faisal, I., Kauppi, L., 2016. Sex chromosome recombination failure, apoptosis, and fertility in male mice. *Chromosoma* 125, 227-235.
- Gajjar, R., Miller, S.D., Meyers, K.E., Ginsberg, J.P., 2015. Fertility preservation in patients receiving cyclophosphamide therapy for renal disease. *Pediatric nephrology (Berlin, Germany)* 30, 1099-1106.
- 435 Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119, 493-501.
- Gillingham, D., Sauter, B., 2017. Genomic Studies Reveal New Aspects of the Biology of DNA Damaging Agents. *ChemBiochem : a European journal of chemical biology* 18, 2368-2375.
- 440 Gligor, V., Gligor, R., 1995. Study of the Cyclophosphamide Teratogenity and Its Influence on the Experimental Animals' Reproduction. *Toxicology Letters* 78, 35.
- Green, D.M., Liu, W., Kutteh, W.H., Ke, R.W., Shelton, K.C., Sklar, C.A., Chemaitilly, W., Pui, C.H., Klosky, J.L., Spunt, S.L., Metzger, M.L., Srivastava, D., Ness, K.K., Robison, L.L., Hudson, M.M., 2014. Cumulative alkylating agent exposure and semen parameters in adult survivors of childhood cancer: a report from the St Jude Lifetime Cohort Study. *The Lancet Oncology* 15, 1215-1223.
- 445 Grenier, L., Robaire, B., Hales, B.F., 2012. The activation of DNA damage detection and repair responses in cleavage-stage rat embryos by a damaged paternal genome. *Toxicol Sci* 127, 555-566.
- 450 Gunes, S., Hekim, G.N., Arslan, M.A., Asci, R., 2016. Effects of aging on the male reproductive system. *Journal of assisted reproduction and genetics* 33, 441-454.
- Harrouk, W., Codrington, A., Vinson, R., Robaire, B., Hales, B.F., 2000. Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. *Mutation Research/DNA Repair* 461, 229-241.
- 455 Herati, A.S., Zhelyazkova, B.H., Butler, P.R., Lamb, D.J., 2017. Age-related alterations in the genetics and genomics of the male germ line. *Fertility and sterility* 107, 319-323.
- Jan, S.Z., Jongejan, A., Korver, C.M., van Daalen, S.K.M., van Pelt, A.M.M., Repping, S., Hamer, G., 2018. Distinct prophase arrest mechanisms in human male meiosis. *Development* 145.
- 460 Kaarouch, I., Bouamoud, N., Madkour, A., Louanjli, N., Saadani, B., Assou, S., Aboulmaouahib, S., Amzazi, S., Copin, H., Benkhalifa, M., Sefrioui, O., 2018. Paternal

- 465 age: Negative impact on sperm genome decays and IVF outcomes after 40 years. *Mol Reprod Dev* 85, 271-280.
- Katz-Jaffe, M.G., Parks, J., McCallie, B., Schoolcraft, W.B., 2013. Aging sperm negatively impacts in vivo and in vitro reproduction: a longitudinal murine study. *Fertility and sterility* 100, 262-268.e261-262.
- 470 Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, J.A., Putman, D.L., Short, J.M., 1991. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. *Proc Natl Acad Sci U S A* 88, 7958-7962.
- 475 Kong, A., Frigge, M.L., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., Gudjonsson, S.A., Sigurdsson, A., Jonasdottir, A., Jonasdottir, A., Wong, W.S., Sigurdsson, G., Walters, G.B., Steinberg, S., Helgason, H., Thorleifsson, G., Gudbjartsson, D.F., Helgason, A., Magnusson, O.T., Thorsteinsdottir, U., Stefansson, K., 2012. Rate of de novo mutations and the importance of father's age to disease risk. *Nature* 488, 471-475.
- Ladstatter, S., Tachibana-Konwalski, K., 2016. A Surveillance Mechanism Ensures Repair of DNA Lesions during Zygotic Reprogramming. *Cell* 167, 1774-1787.e1713.
- 480 Lazarou, S., Morgentaler, A., 2008. The effect of aging on spermatogenesis and pregnancy outcomes. *The Urologic clinics of North America* 35, 331-339, xi.
- Liu, M., Hales, B.F., Robaire, B., 2014. Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. *Biology of reproduction* 90, 72.
- 485 Lowe, X., O'Hogan, S., Moore, D., 2nd, Bishop, J., Wyrobek, A., 1996. Aneuploid epididymal sperm detected in chromosomally normal and Robertsonian translocation-bearing mice using a new three-chromosome FISH method. *Chromosoma* 105, 204-210.
- 490 Maher, G.J., Ralph, H.K., Ding, Z., Koelling, N., Mlcochova, H., Giannoulatou, E., Dhimi, P., Paul, D.S., Stricker, S.H., Beck, S., McVean, G., Wilkie, A.O.M., Goriely, A., 2018. Selfish mutations dysregulating RAS-MAPK signaling are pervasive in aged human testes. *Genome research*.
- 495 Martin, G.M., Smith, A.C., Ketterer, D.J., Ogburn, C.E., Disteche, C.M., 1985. Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. *Israel journal of medical sciences* 21, 296-301.
- 500 Martinez, G., Walschaerts, M., Le Mitouard, M., Borye, R., Thomas, C., Auger, J., Berthaut, I., Brugnol, F., Daudin, M., Moinard, N., Ravel, C., Saias, J., Szerman, E., Rives, N., Hennebicq, S., Bujan, L., 2017. Impact of Hodgkin or non-Hodgkin lymphoma and their treatments on sperm aneuploidy: a prospective study by the French CECOS network. *Fertility and sterility* 107, 341-350.e345.
- Milholland, B., Dong, X., Zhang, L., Hao, X., Suh, Y., Vijg, J., 2017. Differences between germline and somatic mutation rates in humans and mice. *Nature communications* 8, 15183.

- 505 Mohn, G.R., Ellenberger, J., 1976. Genetic effects of cyclophosphamide, ifosfamide and trofosfamide. *Mutation Research/Reviews in Genetic Toxicology* 32, 331-360.
- Oliveira, R.J., Pesarini, J.R., Sparca Salles, M.J., Nakamura Kanno, T.Y., Dos Santos Lourenco, A.C., da Silva Leite, V., da Silva, A.F., Matiazi, H.J., Ribeiro, L.R., Mantovani, M.S., 2014. Effects of beta-glucan polysaccharide revealed by the dominant lethal assay and micronucleus assays, and reproductive performance of male mice exposed to cyclophosphamide. *Genetics and molecular biology* 37, 111-119.
- 510 Paul, C., Robaire, B., 2013. Ageing of the male germ line. *Nature reviews Urology* 10, 227-234.
- Perez-Cerezales, S., Miranda, A., Gutierrez-Adan, A., 2012. Comparison of four methods to evaluate sperm DNA integrity between mouse caput and cauda epididymidis. *Asian journal of andrology* 14, 335-337.
- 515 Pina-Guzman, B., Solis-Heredia, M.J., Rojas-Garcia, A.E., Uriostegui-Acosta, M., Quintanilla-Vega, B., 2006. Genetic damage caused by methyl-parathion in mouse spermatozoa is related to oxidative stress. *Toxicol Appl Pharmacol* 216, 216-224.
- 520 Sailer, B.L., Jost, L.K., Erickson, K.R., Tajiran, M.A., Evenson, D.P., 1995. Effects of X-irradiation on mouse testicular cells and sperm chromatin structure. *Environmental and molecular mutagenesis* 25, 23-30.
- Schmid, T.E., Xu, W., Adler, I.D., 1999. Detection of aneuploidy by multicolor FISH in mouse sperm after in vivo treatment with acrylamide, colchicine, diazepam or thiabendazole. *Mutagenesis* 14, 173-179.
- 525 Selvaratnam, J.S., Robaire, B., 2016. Effects of Aging and Oxidative Stress on Spermatozoa of Superoxide-Dismutase 1- and Catalase-Null Mice. *Biology of reproduction* 95, 60.
- Servitzoglou, M., De Vathaire, F., Oberlin, O., Patte, C., Thomas-Teinturier, C., 2015. Dose-Effect Relationship of Alkylating Agents on Testicular Function in Male Survivors of Childhood Lymphoma. *Pediatric hematology and oncology* 32, 613-623.
- 530 Sladek, N.E., 1988. Metabolism of oxazaphosphorines. *Pharmacol Ther* 37, 301-355.
- Smart, E., Lopes, F., Rice, S., Nagy, B., Anderson, R.A., Mitchell, R.T., Spears, N., 2018. Chemotherapy drugs cyclophosphamide, cisplatin and doxorubicin induce germ cell loss in an in vitro model of the prepubertal testis. *Scientific reports* 8, 1773.
- 535 Stevens, J.M., Claudia, 2008. Acrolein: Sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res* 52, 7-25.
- Szikriszt, B., Poti, A., Pipek, O., Krzystanek, M., Kanu, N., Molnar, J., Ribli, D., Szeltner, Z., Tusnady, G.E., Csabai, I., Szallasi, Z., Swanton, C., Szuts, D., 2016. A comprehensive survey of the mutagenic impact of common cancer cytotoxics. *Genome Biol* 17, 99.
- 540

- Tiseo, B.C., Cocuzza, M., Bonfa, E., Srougi, M., Silva, C.A., 2016. Male fertility potential alteration in rheumatic diseases: a systematic review. *International braz j urol : official journal of the Brazilian Society of Urology* 42, 11-21.
- 545 Trasler, J.M., Hermo, L., Robaire, B., 1988. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. *Biology of reproduction* 38, 463-479.
- Trasler, J.M., Robaire, B., 1988. Effects of cyclophosphamide on selected cytosolic and mitochondrial enzymes in the epididymis of the rat. *J Androl* 9, 142-152.
- 550 Vaisheva, F., Delbes, G., Hales, B.F., Robaire, B., 2007. Effects of the chemotherapeutic agents for non-Hodgkin lymphoma, cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), on the male rat reproductive system and progeny outcome. *J Androl* 28, 578-587.
- 555 Veal, G.J., Cole, M., Chinnaswamy, G., Sludden, J., Jamieson, D., Errington, J., Malik, G., Hill, C.R., Chamberlain, T., Boddy, A.V., 2016. Cyclophosphamide pharmacokinetics and pharmacogenetics in children with B-cell non-Hodgkin's lymphoma. *Eur J Cancer* 55, 56-64.
- Wheeler, G.P., 1962. Studies related to the mechanisms of action of cytotoxic alkylating agents: a review. *Cancer Res* 22, 651-688.
- 560 World Health Organisation, 2010. WHO laboratory manual for the examination and processing of human semen. World Health Organisation,, Switzerland.
- Yatsenko, A.N., Turek, P.J., 2018. Reproductive genetics and the aging male. *Journal of assisted reproduction and genetics* 35, 933-941.
- 565 Yauk, C.L., Lambert, I.B., Meek, M.E., Douglas, G.R., Marchetti, F., 2015. Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement. *Environmental and molecular mutagenesis* 56, 724-750.
- Zhang, X., Ebata, K.T., Robaire, B., Nagano, M.C., 2006. Aging of male germ line stem cells in mice. *Biology of reproduction* 74, 119-124.
- 570 Zhao, Y., Epstein, R.J., 2008. Programmed genetic instability: a tumor-permissive mechanism for maintaining the evolvability of higher species through methylation-dependent mutation of DNA repair genes in the male germ line. *Molecular biology and evolution* 25, 1737-1749.
- 575 Zou, L., 2016. A Zygotic Checkpoint for Unrepaired Lesions. *Cell* 167, 1676-1678.