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The comet assay in male reproductive toxicology

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Abstract Due to our lifestyle and the environment we live in, we are constantly confronted with genotoxic or potentially genotoxic compounds. These toxins can cause DNA damage to our cells, leading to an increase in mutations. Sometimes such mutations could give rise to cancer in somatic cells. However, when germ cells are affected, then the damage could also have an effect on the next and successive generations. A rapid, sensitive and reliable method to detect DNA damage and assess the integrity of the genome within single cells is that of the comet or single-cell gel electrophoresis assay. The present communication gives an overview of the use of the comet assay utilising sperm or testicular cells in reproductive toxicology. This includes consideration of damage assessed by protocol modification, cryopreservation vs the use of fresh sperm, viability and statistics. It further focuses on in vivo and in vitro comet assay studies with sperm and a comparison of this assay with other assays measuring germ cell genotoxicity. As most of the de novo structural aberrations occur in sperm and spermatogenesis is functional from puberty to old age, whereas female germ cells are more complicated to obtain, the examination of male germ cells seems to be an easier and logical choice for research and testing in reproductive toxicology. In addition, the importance of such an assay for the paternal impact of genetic damage in offspring is undisputed. As there is a growing interest in the evaluation of genotoxins in male germ cells, the comet assay allows in vitro and in vivo assessments of various environmental and lifestyle genotoxins to be reliably determined.

Keywords Comet assay · Reproductive toxicology · Sperm

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Introduction

In today's society, we are constantly being exposed to an increasing number of potentially harmful chemicals due to our life style, the environment, medical treatments and our innate susceptibility. Hence, our reproductive cells are of particular concern as they pass on our genetic material to the next generation. The DNA integrity of germ cells thus has crucial importance. In 1984, Ostling and Johanson published a method using micro-gel electrophoresis of immobilised cells lysed at high salt concentrations, which had been embedded in agarose. When they applied an electrophoretic field with pH conditions less than pH 10, tails were observed where the DNA migrated faster than the nuclear DNA (Ostling and Johanson 1984). Alkaline conditions for DNA unwinding and

electrophoresis were incorporated later (Singh et al. 1988), which allowed, at a pH of ≥13, the detection of double- (DSB) and single-strand breaks (SSB) and alkali-labile sites (ALS). This induced damage gave rise to the tail of the comet consisting mainly of single-stranded DNA (Collins et al. 1997). The extensive appliance of the comet assay with its various modifications led to the establishment of guidelines for its use (Tice et al. 2000). These have been very valuable as a basis to standardise protocols when carrying out the comet assay with a variety of cell types and organs either in vivo or in vitro. However, these guidelines are not entirely applicable when investigating reproductive cells like sperm in the comet assay, unless several adjustments are made − particularly to relax the highly compacted sperm chromatin structure. The comet assay has also been carried out with mammalian oocytes (Jebelli et al. 2001) and embryos (Van Blerkom et al. 2001), but this review will focus primarily on the use of the sperm comet assay in reproductive toxicology, as there is a growing interest in the evaluation of genotoxins in male germ cells.

Assessing the DNA damage

The basic principles of the comet assay introduced to the scientific community in the mid-1980s (Ostling and Johanson 1984) were based on previous results, which characterised the nuclear structure of lysed cells as containing superhelical DNA (Cook et al. 1976). Furthermore, cells being treated with high concentrations of salt in the presence of a non-ionic detergent only remained as the nuclear scaffold comprised of RNA and proteins together with the attached looped DNA (Rydberg and Johanson 1978). The alkaline denaturation and the separation of the DNA double helix seemed to be an important step in detecting DNA damage (Singh et al. 1988) allowing the detection of mainly SSB and ALS. Collins et al. (1997) suggested that the formation of the comet tail seemed to originate predominantly from relaxation of supercoiled loops, rather than alkaline unwinding. Nevertheless, unwinding occurs under alkaline conditions, and single-stranded DNA can be observed in the comet tail (Collins et al. 1997). By choosing different pH conditions for electrophoresis and the preceding incubation, different damage types and different levels of sensitivity can be assessed. Under neutral (pH 8–9) conditions, mainly DSB are detected, although some SSB due to the relaxation of supercoiled loops containing the breaks might also contribute to the observed comet (Collins 2004). Alternatively, under alkaline conditions, DSB and SSB (at pH 12.3) and additionally ALS (at pH≥13) can be visualised resulting in increased DNA migration in the electrophoretic field (Fairbairn et al. 1995).

The neutral comet assay allows the detection of almost exclusively DSB by merely subjecting lysed cell nuclei to an electrophoretic field at neutral pH (Haines et al. 1998; Singh and Stephens 1998). Under these neutral electrophoresis conditions, the amount of DNA damage seems to be reduced when compared to alkaline conditions, which is probably due to either an alkaline environment being required to reveal certain DNA lesions and/or the migration of damaged DNA being greater at a higher pH (Angelis et al. 1999). Depending on the genotoxin, the amount of DNA damage in the neutral assay might also be lower as only a few agents besides ionising radiation induce DSB (Lundin et al. 2005). In the absence of DNA damaging agents, the neutral comet assay seems to be rather sensitive to DNA loop relaxation, which was shown with certain intercalating agents, e.g., ethidium bromide, which is able to hyper-condense DNA in lysed cells below control level (Belyaev et al. 1999). In general, with the neutral comet assay, the vast majority of SSB will not be detected unless the DNA is unwound, i.e., made single stranded under alkaline conditions. High pH conditions (above pH 13) then allow also the detection of ALS. Therefore, the sensibility of the comet assay towards certain types of induced DNA damage can be increased by carefully choosing the pH conditions.

When using endonuclease III or similar repair enzymes as an intermediate step in the comet assay (Collins et al. 1993; Moller 2006; Smith et al. 2006), oxidised pyrimidines can be converted into SSB (Aukrust et al. 2005). This modified comet assay is currently used as a biomarker for oxidative DNA damage (Albertini et al. 2000; Collins et al. 1993; Hwang and Kim 2007). Many toxicants do not cause primarily strand breaks but damage the DNA by cross-linking (Henderson et al. 1998) or introducing AP (apurinic or apyrimidinic) sites (Bilbao et al. 2002), which are alkali-labile and will be developed to SSB under alkaline conditions. Intermediates

and base-free positions in the DNA introduced by glycosylases through base excision repair (Olsen et al. 2001) are just two examples for detectable ALS. A high innate activity of enzymes involved in rapid excision repair, however, can create high levels of incision-related breaks in the comet assay and therefore lead to a larger comet tail (Collins et al. 1995). Cross-linked DNA, in contrast to other DNA damage, inhibits DNA migration in the electrophoretic field by stabilising the DNA (Merk and Speit 1999; Pfuhler and Wolf 1996).

Using image analysis in combination with the comet assay, apoptotic or necrotic cells can also be identified due to their small or non-existent head and large diffuse comet tails, so-called ghosts or "hedgehogs" – comet images of lymphocytes with excessive damage (Olive and Banath 1995). For sperm, such highly damaged cells showing extensive DNA fragmentation cannot be excluded (Sakkas et al. 2003). There is still an ongoing debate about these ghost cells arising from apoptotic events. These comet images showing extensive damage seem not to originate from early stages of apoptotic events but rather from dead cells. The extensive fragmentation into low molecular weight DNA fragments seem to be associated with late apoptotic events (Czene et al. 2002). Thus, it has been suggested that comet images cannot be used for the interpretation of induced genotoxic DNA damage when a risk from apoptosis is present (Choucroun et al. 2001). Other findings, on the other hand, suggest that results obtained with the comet assay are not confounded by concomitant processes leading to apoptosis (Roser et al. 2001). The mutagen-induced DNA damage measured with the comet assay appears to be the principal cause for the induced damage (Rundell et al. 2003), making the comet assay a valuable tool for evaluating agent-induced DNA damage. However, when late apoptotic events are intentionally targeted with a highly sensitive method like the comet assay, it has been suggested omitting the electrophoresis step for quantifying the late apoptotic fraction (Godard et al. 1999).

The use of sperm and testicular cells in the comet assay

To collect toxicological data for genotoxins that target and affect the reproductive system, it is necessary to examine germ cells for DNA damage and DNA integrity. It is not sufficient to study more easily accessible surrogate cells (Albertini et al. 2000). Mature germ cells have to be used for the evaluation of reproductive genotoxicants. Sperm have several major advantages when compared to other reproductive cells: provided ethical approval has been obtained, sampling is non-invasive and fairly easy, and quite a large number of cells can be collected at one sampling time. Most importantly, the male reproductive system is constantly producing sperm throughout most of the life span of an individual. During mammalian spermiogenesis, the last part of spermatogenesis, the haploid spermatocytes undergo major morphological changes when the genome is repacked and compacted. Besides the loss of most of the cytosol and the development of a tail, the spermatids' nuclear chromatin condenses to a very dense, crystalline-like structure facilitated by protamines. Protamines stabilise the DNA covalently by intra- and intermolecular disulfide bonds (Balhorn 1982; Chapman and Michael 2003). During this condensation process, about 85% of the histone-bound DNA in human sperm will be transformed into compact nucleoprotamine chromatin (Wykes and Krawetz 2003).

Therefore, utilising sperm for the comet assay requires decondensation of the highly condensed DNA before lysis, unwinding and electrophoresis (Singh et al. 1989). Using alkaline conditions at pH≥13 in the comet assay, human and mouse sperm yield surprisingly high amounts of SSB (10^6–10^7 per genome) mostly due to ALS. Neither human lymphocytes under alkaline conditions nor sperm under neutral conditions show these DNA breaks, which may represent functional characteristics but not pre-existing SSB (Singh et al. 1989). In addition to intracellular processes like DNA replication and DNA repair, which utilise DNA nicking enzymes (Fairbairn et al. 1994; McPherson and Longo 1993) during spermatogenesis, the reason for this high number of ALS seems to be the high degree of chromatin condensation (Fernandez et al. 2000; Singh et al. 1989). Under alkaline conditions, nicks in the DNA seem to provide a starting point for DNA unwinding by transforming the breaks into single strands (Rydberg 1975; Vazquez-Gundin et al. 2000). Published data suggest that sperm nuclei compared to leucocytes contain more than double the amount of SSB (Muriel et al. 2004). More importantly,

sperm chromatin is ninefold more enriched in single-stranded segments – potentially prone to becoming ALS. Due to the higher degree of compaction, those partially denatured sections maybe the result of elevated torsional stress of DNA loops (Muriel et al. 2004). Impressively, mammalian sperm chromatin is six times more highly compacted than metaphase chromosomes, although it seems to be organised very specifically (Ward and Coffey 1991). The susceptibility of human sperm to alkaline DNA denaturation seems to be strongly correlated with DNA strand breaks indicating an important physiological relevance of sperm quality and fertility (Aravindan et al. 1997). Fertile sperm tend to be more resistant to agent-induced DNA breakage than infertile samples (McKelvey-Martin et al. 1997). This fact makes carrying out semen analysis according to World Health Organisation (WHO) criteria (WHO 1999) and completing a reproductive questionnaire for the donors, an absolute prerequisite. By taking into account that mature sperm do not have DNA repair capacity (van Loon et al. 1993), three potential mechanisms, which may be independently or co-dependently accountable for basic sperm damage, have been identified (Trisini et al. 2004): (1) defective chromatin condensation during spermiogenesis (Manicardi et al. 1995; McPherson and Longo 1993; Sailer et al. 1995), (2) apoptotic events during spermatogenesis, epididymal maturation or in the ejaculate (Gorczyca et al. 1993; Sakkas et al. 1999) and (3) oxidative stress from reactive oxygen species (de Lamirande and Gagnon 1998; Irvine et al. 2000).

Nonetheless, the comet assay with human sperm is able to identify low levels of DNA damage (Anderson et al. 1996; Hughes et al. 1997; Irvine et al. 2000; Singh and Stephens 1998; Sun et al. 1997) even if the scored comet images are more heterogeneous than those seen with lymphocytes. The preferred comet parameter to describe the observed DNA damage is the tail moment or olive tail moment, which provides the most stable estimates for DNA damage because it has a larger degree of uniformity in quantile dispersions (Lee et al. 2004). However, for sperm, additionally, the percent head DNA is used due to the high background levels of SSB of about 20% head DNA (Hughes et al. 1996; McKelvey-Martin et al. 1997).

Based on the protocol of Singh et al. (1988, 1989), various groups have adjusted the basic method of single-cell gel electrophoresis (SCGE) for the use of human or animal sperm. Essential to the recent protocols used (Anderson et al. 2003; Baumgartner et al. 2004; Duty et al. 2003; Haines et al. 2002; Irvine et al. 2000; Tomsu et al. 2002) is that sperm are immobilised within a layer of low-melting point agarose (0.5–1%) and spread out onto dry agarose-coated slides. An optional cover layer of agarose may serve as protection of the cell-containing layer. Subsequently, in the lysis step, cell membrane, cytosol and nuclear membrane are removed via incubation in a lysing buffer (100 mM EDTA, 10 mM Tris, pH 10) containing a high concentration of salt (2.5 M NaCl) and a non-ionic detergent (1% Triton X-100; Singh et al. 1988). The use of 10% dimethyl sulphoxide (DMSO) in the lysing solution varies; however, it can be added as a protectant against free radicals within the lysing solution. Because of the highly compacted sperm, chromatin human sperm nuclei are decondensed by incubation in 4–10 mM dithiothreitol (DTT; Incharoensakdi and Panyim 1981; Singh et al. 1989) and/or 0.05-0.1 mg/ml proteinase K (PK; Perreault and Zirkin 1982) or 10 mg/ml RNase (Duty et al. 2003). The decondensation procedure in sperm is known to vary in temperature, length and strength of incubation depending on the species, e.g., for mice (Baulch et al. 2007). After lysis and equilibration in electrophoresis buffer, the agarose-embedded nuclei are subject to electrophoresis. Electrophoresis can be performed with different pH conditions achieving detection of different sensitivities of DNA damage (Angelis et al. 1999). The layer of buffer above the slides should be around 1–2 mm resulting in a current of approximately 300 mA (alkaline buffer, pH≥13) at 0.7–0.9 V/cm. Depending on the pH of the electrophoresis buffer, a neutralisation step is necessary. To stain the nuclei for microscopic analysis, various fluorescent dyes can be used (ethidium bromide, YOYO, SYBR-Green). It should be mentioned that the comet assay has also been successfully applied to previously dried and methanol-fixed sperm before applying the agarose (Chan et al. 2001; Connelly et al. 2001).

Cryopreserved vs fresh sperm

It is more practical in larger studies to evaluate the DNA integrity of sperm in cryopreserved semen rather than fresh sperm (Duty et al. 2002); however, freezing living cells can cause unfavourable and damaging effects due to ice crystal formation and/or

severe osmotic changes. Freezing seems to affect chromatin structure and sperm morphology (Hammadeh et al. 1999), and DNA damage from cryopreservation in semen from infertile men has been detected using the alkaline comet assay (Donnelly et al. 2001a). Additionally, the sperm chromatin structure assay (SCSA) also revealed that the sperm quality deteriorated upon cryopreservation (Gandini et al. 2006). Cryopreservation of testicular spermatozoa by itself may reduce pregnancy rates (Thompson-Cree et al. 2003). The freezing–thawing process affects the DNA integrity of boar spermatozoa when assessing post-thaw quality of boar semen using the neutral comet assay (Fraser and Strzezek 2005) and SCSA (Hernandez et al. 2006). Conventional cryopreservation and storage in liquid nitrogen caused DNA damage in thawed macaque sperm, even more without cryoprotectants, but with the exception of the motile sperm fraction (Li et al. 2007). However, a vital role for the integrity of the cell membrane is the way of freezing sperm in terms of speed, step-wise changes in temperature and the cryopreservative used (Gilmore et al. 2000; Morris et al. 1999). In another study, no differences were found with the comet assay when fresh and frozen human sperm were compared (Steele et al. 2000). For the use of sperm with the comet assay in reproductive toxicology, flash-freezing in liquid nitrogen seems to be the method-of-choice, which most closely reproduced results obtained with fresh sperm (Duty et al. 2002). In addition, freezing sperm in seminal plasma only improves post-thaw motility and DNA integrity (Donnelly et al. 2001b).

Viability considerations

For the comet assay, it is imperative to test cells for viability after treatment to exclude cytotoxic effects as untreated cells show background levels of DNA damage of around 0–10% DNA in the tail depending on the cell type (Collins 2004) and approximately 20% DNA in the tail for sperm (McKelvey-Martin et al. 1997). A viability of greater than 75% should be produced for the maximum concentration of a tested compound to avoid a false positive response due to cytotoxicity (Henderson et al. 1998). Recently, it was suggested that, when only 50 cells are being scored, cell viability should be above 95% (Singh 2000). It should also be noted that it is not feasible to measure cell viability on cells from solid tissues due to the disruption of the cell membranes when separating the cells (Singh 1998). A variety of viability tests for the vitality of germ cells exist and are also used for sperm like the Trypan blue exclusion test (Anderson et al. 2003; Aslam et al. 1998; Talbot and Chacon 1981). As this exclusion test only indicates an intact membrane and not necessarily a viable cell, genuine viability tests like the dual fluorescence method with SYBR-14/propidium iodide staining are more suitable (Ferrara et al. 1997; Garner and Johnson 1995). Especially for sperm, viability can vary and is considered normal under WHO criteria when above 50% for Trypan blue exclusion and >60% for the hyper-osmotic swelling test (WHO 1999, Appendices IA and IV). It has also been shown that freezing sperm increases the rigidity of the membrane and decreases the viability to values of 45% using an eosin–nigrosin viability test (Giraud et al. 2000). Thus, for the in vitro sperm comet assay, it is perhaps necessary to determine cytotoxicity of chemicals in parallel via the viability of lymphocytes according to published guidelines (Collins 2004) to ensure against possible artefacts.

Statistical analysis

The number of sperm cells evaluated with the comet assay must represent a balance between accuracy and precision. It has been suggested in the comet guidelines to blindly score on independently coded slides at least 50 cells per culture or individual with 25 cells scored per duplicate (Tice et al. 2000). However, published studies display a large variability of approaches to the number of cells scored. For instance, 100 cells are scored on three independent repeats (Yamauchi et al. 2007), 100 cells per sample (based on 2 duplicates; Bian et al. 2004) and 150 cells per sample (50 cells per slide; Ambrosini et al. 2006).

It is crucial to understand the hierarchy that accounts for many experimental designs where a number of cells are scored on a number of slides for each sample and that the sample rather than the cell is the unit for statistical analysis (Lovell et al. 1999). Otherwise, the degree of freedom will vary highly and result in a type-2 statistical error. It also has to be taken into consideration that in vivo experiments can result in lower sensitivity than expected due to the

internal variability within the groups. Despite the higher sensitivity observed in vitro, variability might be overlooked if no repeats or duplicates have been included in the experimental design. It is important to identify an appropriate number of individuals for in vivo studies or repeat experiments in vitro to perform a suitable statistical analysis. Finally, it is worth mentioning that, in addition to the statistical significance of a finding, it is necessary to understand and interpret the biological relevance of the data obtained.

In vivo comet assay with sperm

The comet assay is a well-established biomarker system for in vivo biomonitoring of occupational exposures. It is able to rapidly and sensitively test DNA-damaging genotoxins and confounding factors influencing responses (Moller et al. 2000). The alkaline version of the comet assay with a pH≥13 has become a reliable and an accepted assay for in vivo genotoxicological evaluations and has been approved by the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and Environment and US Food and Drug Administration (Burlinson et al. 2007), due to the development of standardised protocols (Collins 2004; Hartmann et al. 2003; McKelvey-Martin et al. 1993; Tice et al. 2000). In the regulatory setting, the comet assay is primarily employed as a very useful follow-up or supplementary in vivo test for mutagenic compounds, which have been shown to produce a positive response in in vitro mutagenicity tests and a negative response in the bone marrow assay, as it demonstrates several advantages over other in vivo indicator tests that are generally accepted. The comet assay can be applied to virtually any organ and differentiated cell type (local genotoxicity), provided an acceptable and suitable cell preparation method exists, and it covers a broader spectrum of primary DNA lesions by evaluating single cells (Brendler-Schwaab et al. 2005). It has been recommended (Hartmann et al. 2003) that 100 to 150 cells per individual have to be evaluated in the in vivo comet assay applications, depending on the number of individuals per group. At least two dose levels are required to be tested: a high dose, which produces signs of toxicity, and a low dose (25–50% of the high dose). Due to undergoing rapid DNA repair in cells other than mature sperm, e.g. spermatocytes, SSB of primary lesions may only be short-lived. Therefore, this kind of DNA damage could be missed by inadequate sampling times (Brendler-Schwaab et al. 2005).

Various studies facilitating the in vivo comet assay on sperm or testicular cells have been done to toxicologically evaluate reprotoxins and genotoxins (see Table 1 for a complete overview). A variety of toxicants has been investigated in vivo in mice including vanadium (Altamirano-Lozano et al. 1996; Leopardi et al. 2005), herbicides like bentazon (Garagna et al. 2005) and X-rays (Cordelli et al. 2003; Dobrzynska 2005; Haines et al. 2001, 2002). In rats, chemotherapeutic drugs like cyclophosphamide (Anderson et al. 1996; Codrington et al. 2004) and bleomycin either on its own (Anderson et al. 1996) or in combination with etoposide and cis-platin (Delbes et al. 2007) have been tested with the comet assay on testicular cells and sperm. Furthermore, chemicals like ethyl methanesulphonate and the testicular toxin ethylene glycol monomethyl ether (Anderson et al. 1996) also have been examined. The in vivo comet assay has also been used with human sperm for evaluating DNA sperm damage of occupational exposure of workers to toxicants like acrylonitrile (Xu et al. 2003), phthalates (Duty et al. 2003) and pesticides such as fenvalerate (Bian et al. 2004) or monitoring populations for environmental exposure to carbaryl and chlorpyrifos, both pesticides, which appeared to be associated with increased DNA damage in human sperm (Meeker et al. 2004). When monitoring populations, it became evident that a positive correlation between age and caffeine intake and DNA damage could be observed in sperm (Schmid et al. 2007). Regression analysis showed that DNA damage was positively associated with age (29–44 years), abnormal sperm and motility and negatively associated with sperm concentration (Morris et al. 2002).

It has been long known that the baseline DNA damage in human and mouse sperm in the comet assay is high when compared to somatic cells due to the presence of ALS (Singh et al. 1989). In addition, ejaculated sperm DNA is significantly more damaged than testicular sperm DNA (Steele et al. 2000). Studies comparing baseline DNA damage in sperm from normozoospermic fertile, normozoospermic infertile and asthenozoospermic infertile did not show a significant difference between the three groups. However, after challenge with X-rays and hydrogen peroxide, it was concluded that the asthenozoospermic infertile group is more susceptible to damage than the

Table 1 In vivo comet assay responses in germ cells

Publication	Comet assay	Species	Targeted cells	Toxicant
Abu-Hassan et al. 2006	N	Human	Sperm	Untreated
Agbaje et al. 2007	A1	Human	Sperm	Untreated
Altamirano-Lozano et al. 1996	A1	Mouse	Testicular cells	Vanadium pentoxide
Anderson et al. 1996	A1	Rat	Testicular cells	Cyclophosphamide, ethyl methanesulphonate, bleomycin, ethylene glycol monoethyl ether
Aravindan et al. 1997	A3	Human	Sperm	Untreated
Banks et al. 2005	A2	Mouse	Sperm	Heat
Barber et al. 2006	A1	Mouse	Sperm	Untreated F1 generation
Baulch et al. 2007	N2	Mouse	Sperm	γ-rays
Belcheva et al. 2004	A	Human	Sperm	Cigarette Smoke
Bertolla et al. 2006	A1	Human	Sperm	Untreated
Bian et al. 2004	A3	Human	Sperm	Fenvalerate
Brinkworth et al. 1998	A1	Mouse	Sperm	1,3-butadiene
Bustos-Obregon and Goicochea 2002	A1	Earthworm	Male germ cells	Parathion
Chatterjee et al. 2000	N2	Human	Sperm	Fludarabine
Cho et al. 2003	A1	Mouse	Sperm	Untreated
Codrington et al. 2004	A2/N3	Rat	Sperm	Cyclophosphamide
Cordelli et al. 2003	A1	Mouse	Testicular cells	X-rays
Delbes et al. 2007	A2/N3	Rat	Sperm	Bleomycin, etoposide, cis-platinum
Ding et al. 2003	=	Human	Sperm	Hypothermia
Dobrzynska et al. 2005	A1	Mouse	Sperm	X-rays, vincristine
Dobrzynska 2005	A1	Mouse	Sperm	X-rays, cyclophosphamide, mitomycin C
Donnelly et al. 2000a	A1	Human	Sperm	Untreated
Duty et al. 2003	N1	Human	Sperm	Phthalates
Garagna et al. 2005	A1	Mouse	Sperm	Bentazon
Haines et al. 2001	N2	Mouse	Sperm	X-rays, Indium-114m
Haines et al. 2002	N2	Mouse	Sperm	X-rays
Hauser et al. 2003	N1	Human	Sperm	PCBs, HCB, DDT, DDE
Hauser et al. 2007	N1	Human	Sperm	Phthalate
Hong et al. 2005	=	Mouse	Testicular cells	50Hz electromagnetic fields
Hughes et al. 1997	A1	Human	Sperm	Untreated
Irvine et al. 2000	A2	Human	Sperm	Untreated
Kotlowska et al. 2007	N2	Turkey	Sperm	Untreated
Labbe et al. 2001	A1	Fish	Sperm	Untreated
Laberge and Boissonneault 2005	A2, N2	Mouse	Spermatids	Untreated
Larson et al. 2001	N1	Human	Sperm	Untreated
Legue et al. 2001	A1	Mouse	Testicular cells	X-rays, interleukins
Leopardi et al. 2005	A2	Mouse	Sperm	Sodium *ortho*-vanadate
Lewis et al. 2004	A1	Human	Sperm	Untreated
Li et al. 2006a	=	Rat	Sperm	Cumene hydroperoxide
Lu et al. 2002	=	Human	Sperm	Untreated
Marty et al. 1999	N1	Mouse	Sperm	Untreated
McVicar et al. 2004	N3	Human	Sperm	Untreated
Meeker et al. 2004	N1	Human	Sperm	Chlorpyrifos, carbaryl
Migliore et al. 2002	A2	Human	Sperm	Styrene
Migliore et al. 2006	A2	Human	Sperm	Styrene
Morris et al. 2002	N2	Human	Sperm	Untreated
Nasr-Esfahani et al. 2005	A1	Human	Sperm	Untreated
O'Connell et al. 2002a	A1	Human	Sperm	Untreated

Table 1 (continued)

Publication	Comet assay	Species	Targeted cells	Toxicant
O'Connell et al. 2002b	A1	Human	Sperm	Untreated
O'Connell et al. 2003	A1	Human	Sperm	Untreated
O'Donovan 2005	A1	Human	Sperm	Chemotherapy
Olsen et al. 2003	A1	Human, rat	Testicular cells	Untreated
O'Neill et al. 2007	A1	Human	Sperm	Untreated
Osipov et al. 2002	=	Mouse	Sperm	γ-rays
Sakkas et al. 2002	A3, N3	Human	Sperm	Untreated
Samanta et al. 2004	A1	Mouse	Testicular cells	X-rays, RP-1 herb extract (*Podophyllum hexandrum*)
Schmid et al. 2003	A1	Human	Sperm	Untreated
Schmid et al. 2007	A1, N2	Human	Sperm	Untreated
Shen and Ong 2000	A3	Human	Sperm	Untreated
Singh et al. 1989	A, N1	Human, mouse	Sperm	Untreated
Singh et al. 2003	N1	Human	Sperm	Untreated
Song et al. 2005	=	Human	Sperm	Benzene
Steele et al. 1999	A1	Human	Sperm	Untreated
Tomsu et al. 2002	A1	Human	Sperm	Untreated
Trisini et al. 2004	N1	Human	Sperm	Untreated
Verit et al. 2006	A2	Human	Sperm	Untreated
Villani et al. 2007	A1	Mouse	Testicular cells	Vanadyl sulphate
Xu et al. 2003	A3	Human	Sperm	Acrylonitrile
Xu et al. 2007	=	Human	Sperm	Untreated
Zhang et al. 2001	=	Mouse	Testicular cells	Smoking
Zhang et al. 2006	=	Mouse	Testicular cells	Lead acetate, vitamin C, thiamin

These publications report the in vivo use of the Comet assay on male germ cells, sperm and testicular cells. Electrophoresis pre-incubation (unwinding) and electrophoresis conditions: alkaline A (pH 10–13), A1 (pH≥13), A2 (pH 12–12.5), A3 (pH 10-11.9); neutral N (pH 7–9), N1 (pH 9), N2 (pH 8–8.5), N3 (pH 7–7.5); a separation by "/" indicates that solutions of different pH have been used for the pre-incubation step and the electrophoresis; "=" indicates that the information is not accessible because the publication is printed in Chinese or Russian.

normozoospermic infertile group, which in turn is more susceptible than the fertile group. The fertile group contains a resistant subpopulation of spermatozoa with relatively intact DNA (Hughes et al. 1996; McKelvey-Martin et al. 1997). Irvine et al. (2000) stated that a significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa. Highly significant negative correlations were observed between DNA fragmentation and semen quality, particularly sperm concentration. In addition, multiple regression analysis indicated that other attributes of semen quality, such as sperm movement and ROS generation, were also related to DNA damage (Irvine et al. 2000). Verit et al. (2006) did not find any relationship between sperm DNA damage and oxidative stress in normozoospermic infertile men and considered that the pathophysiology of idiopathic infertility cannot be explained by sperm DNA damage or seminal oxidative stress (Verit et al. 2006). Trisini et al. (2004) attempted to find associations between semen parameters and sperm DNA damage with the neutral comet assay (comet extent and tail moment). Although there were associations between semen and comet assay parameters, their magnitudes were weak, suggesting that the comet assay provides additional independent information on sperm function.

In vitro comet assay with sperm

Studies with the in vitro comet assay on sperm (see Table 2 for a complete overview) have mainly been focusing on the investigation of the potential genotoxic damage of compounds such as flavonoids (silymarin, myricetin, quercetin, kaempferol, rutin and kaempferol-3-rutinoside) and food mutagens [3-amino-1-methyl-5H-pyrido (4,3-b)indole (Trp) and

Table 2 In vitro Comet assay responses in germ cells

Publication	Comet assay	Species	Targeted cells	Toxicant
Aitken et al. 1998	A2	Human	Sperm	Hydrogen peroxide
Ambrosini et al. 2006	A1	Human	Sperm	Oleoylethanolamide
Anderson et al. 1997a	A1	Human	Sperm	Oestrogens, dibromochloropropane, butadiene metabolites
Anderson et al. 1997b	A1	Human	Sperm	Oestrogens, lead, dibromochloropropane, ethylene glycol monoethyl ether, butadiene metabolites
Anderson et al. 1997c	A1	Human	Sperm	Trp, IQ, antioxidants
Anderson et al. 1997d	A1	Mouse, rat	Testicular cells	Butadiene metabolites
Anderson et al. 1998	A1	Human	Sperm	Trp, IQ, PhiP, flavonoids
Anderson et al. 2003	A1	Human	Sperm	Hydrogen peroxide, SOD, catalase, vitamin C
Arabi and Heydarnejad 2007	=	Boar	Sperm	Cadmium
Arabi 2004	A2	Human	Sperm	Nicotine
Arabi 2005	A1	Bull	Sperm	Mercury chloride
Atorino et al. 2001	A3	Rat	Testicular cells	Hydrogen peroxide, γ-rays
Baumber et al. 2003	A1	Horse	Sperm	Xanthine, xanthine oxidase, catalase, SOD, glutathione
Baumber et al. 2005	A1	Horse	Sperm	Vitamins C&E, catalase, SOD, glutathione
Baumgartner et al. 2004	A1	Human	Sperm	Doxorubicin
Bjorge et al. 1995	A1	Rat	Testicular cells	1,2-Dibromo-3-chloropropane
Bjorge et al. 1996	A1	Human, rat	Testicular cells	1,2-Dibromo-3-chloropropane, 4-nitroquinoline N-oxide, X-rays
Boe-Hansen et al. 2005	N2	Bull	Sperm	Mechanical stress
Cabrita et al. 2005	A2	Fish	Sperm	Freezing
Cemeli et al. 2004	A1	Human	Sperm	Hydrogen peroxide, oestrogen-like compounds, flavonoids
Chan et al. 2001	N3	Human	Sperm	Hydrogen peroxide
Chen et al. 2002	A1, N1	Hamster	Sperm	NADPH
Chen et al. 2003	A1	Hamster	Sperm	NADPH, SOD
Ciereszko et al. 2005	A1, N2	Fish	Sperm	Hydrogen peroxide, UV, bisazir
Connelly et al. 2001	N3	Human	Sperm	E6-E7 HPV DNA fragments
Dietrich et al. 2005	A1	Fish	Sperm	Hydrogen peroxide, UV
Ding et al. 2004	=	Human	Sperm	Microwave radar
Dobrzynska et al. 2004	A1	Human	Sperm	Triiodothreonine, L-thyroxine, noradrenaline
Donnelly et al. 1999	A1	Human	Sperm	Vitamins C & E, hydrogen peroxide
Donnelly et al. 2000b	A1	Human	Sperm	Hydrogen peroxide, glutathione, hypotaurine
Donnelly et al. 2001a	A1	Human	Sperm	Freezing
Donnelly et al. 2001b	A1	Human	Sperm	Freezing
Duty et al. 2002	N1	Human	Sperm	Freezing
Fraser and Strzezek 2004	N2	Boar	Sperm	Freezing
Fraser and Strzezek 2005	N1	Boar	Sperm	Freezing
Fraser and Strzezek 2007a	N1	Boar	Sperm	Freezing
Fraser and Strzezek 2007b	N1	Boar	Sperm	Freezing
Garagna et al. 2001	A1	Mouse, rabbit	Sperm	TCDD
Gloor et al. 2006	N1	Cat	Sperm	X-ray, freezing
Gwo et al. 2003	A3	Oyster	Sperm	Freezing
Haines et al. 1998	A, N2	Mouse	Sperm	γ-rays
Huang et al. 2003	=	Mouse	Testicular cells	Hydrogen peroxide, *lycium barbarum* polysaccharides
Hughes et al. 1996	A1	Human	Sperm	Hydrogen peroxide, X-rays
Hughes et al. 1998	A1	Human	Sperm	Vitamins C & E, urate, acetyl cystein, X-rays

Table 2 (continued)

Publication	Comet assay	Species	Targeted cells	Toxicant
Hughes et al. 1999	A1	Human	Sperm	Vitamins C & E, X-rays
Jiang et al. 2007	A3[a]	Boar	Sperm	Freezing
Labaj et al. 2004[b]	A1	Rat	Testicular cells	Lignin, hydrogen peroxide, N-methyl-N'-nitrosoguanine
Lazarova et al. 2004[b]	A1	Rat	Testicular cells	Carboxymethyl chitin-glucan, hydrogen peroxide
Lazarova et al. 2006a[b]	A1	Rat	Testicular cells	Carboxymethyl chitin-glucan, methylene blue
Lazarova et al. 2006b	A1	Rat	Testicular cells	Hydrogen peroxide, N-nitrosomorpholine, methylene blue, benzo[a]pyrene
Li et al. 2006b	N2	Human	Sperm	Hydrogen peroxide
Li et al. 2007	A1	Monkey	Sperm	Freezing
Linfor and Meyers 2002	A1	Horse	Sperm	Freezing
McKelvey-Martin et al. 1997[b]	A1	Human	Sperm	X-rays, hydrogen peroxide
Morse-Gaudio and Risley 1994	A1	Frog	Testicular cells	Teniposide (VM-26)
Olsen et al. 2001	A1	Human, rat	Testicular cells	Methylmethane sulfonate
Perrin et al. 2007	A1	Rat	Cultured spermatocytes	γ-rays
Sierens et al. 2002	A2	Human	Sperm	Hydrogen peroxide, isoflavones, vitamins C & E
Singh and Stephens 1998	N	Human	Sperm	X-rays
Song et al. 2002	=	Human	Sperm	Freezing
Steele et al. 2000	A1	Human	Sperm	Freezing
Tang and Xuan 2003	=	Mouse	Sperm	Carbon disulfide
Thompson-Cree et al. 2003	A1	Human	Sperm	Freezing
Van Kooij et al. 2004	N	Human	Sperm	X-ray
Wellejus et al. 2004	A1	Rat	Testicular cells	17alpha-ethinylestradiol
Xu et al. 2000	A3	Human	Sperm	Hydrogen peroxide
Yang et al. 2004	=	Human	Sperm	Sodium nitroprusside, zinc
Young et al. 2003	N1	Human	Sperm	Holding at RT
Zheng and Olive 1997[b]	A	Mouse	Testicular cells	X-rays
Zhou et al. 2006[b]	A1	Fish	Sperm	Duroquinone
Zilli et al. 2003	A2	Fish	Sperm	Freezing

These publications report the in vitro use of the Comet assay on male germ cells, sperm and testicular cells. Electrophoresis pre-incubation (unwinding) and electrophoresis conditions: alkaline A (pH 10–13), A1 (pH≥13), A2 (pH 12–12.5), A3 (pH 10–11.9); neutral N (pH 7–9), N1 (pH 9), N2 (pH 8-8.5), N3 (pH 7–7.5); a separation by "/" indicates that solutions of different pH have been used for the pre-incubation step and the electrophoresis; "=" indicates that the information is not accessible because the publication is printed in Chinese or Russian.

[a] This publication states the use of the neutral Comet assay; however, within this list, the assay is grouped into the alkaline Comet assay because of the pH 10 buffer used for unwinding and electrophoresis.

[b] These publications are using both in vivo and in vitro treatment.

2-amino-3-methylimidazo-4,5-f)quinoline (IQ)] either on their own or in combination (Anderson et al. 1997c). Further research has been carried out on oestrogens (diethylstilbestrol, beta-estradiol, daidzein, genestein, equol and nonylphenyl) either on their own (Anderson et al. 1997b), combined with antioxidants (catalase, vitamin C and SOD; Anderson et al. 2003), or combined with flavonoids (quercetin, kaempferol; Cemeli et al. 2004). Other toxicants investigated included X-rays (Singh and Stephens 1998), gamma radiation (Haines et al. 1998), doxorubicin (Baumgartner et al. 2004), lead sulphate, nitrate and acetate, dibromochloropropane, ethylene glycol monoethyl ether, 1,2-epoxybutene and 1,2,3,4-diepoxybutane (Anderson et al. 1997b). All compounds produced positive responses, but ethylene

glycol monoethyl ether only produced positive responses in sperm and not in peripheral lymphocytes, and similarly, the phytoestrogens, genistein and daidzein were less responsive in the peripheral lymphocytes in the male than in the sperm. This may be due to greater sensitivity of mature spermatozoa because of their lack of repair (Anderson et al. 1997b).

However, as damage was generally seen over a similar dose range, a one-to-one or a one-to-two ratio of somatic and germ cell damage was observed, and this has implications for man for risk assessment purposes (Anderson et al. 1997b; Anderson et al. 1997c). It was later concluded that human testicular cells have limited capacity to repair important oxidative DNA lesions, which could lead to impaired reproduction and de novo mutations (Olsen et al. 2003). By contrast, the usefulness of in vitro cultures of rat spermatocytes and Sertoli cells in conjunction with the comet assay has been reported. This revealed the presence of DNA strand-breaks in non-treated cells, whose numbers decreased with the duration of the culture, suggesting the involvement of DNA repair mechanisms related to meiotic recombination (Perrin et al. 2007). Besides repair capacity, it should also be taken into account that when using cells from testes for in vitro studies, various testicular cell types show differences in metabolic activation of chemical compounds (Bjorge et al. 1995). Anderson et al. (1997c) believe that there are low levels of metabolic activity even in sperm because the heterocyclic amines normally requiring metabolic activation have shown positive responses.

The sperm comet assay versus other assays used in reproductive toxicology

As sperm integrity is essential for successful fertilisation and the subsequent embryo development (Erenpreiss et al. 2006), several assays on spermatozoa have been developed in the last few years to evaluate DNA integrity and to determine DNA fragmentation (Fraser 2004). These include the sperm chromatin dispersion (SCD) and the DNA-breakage detection fluorescence in situ hybridisation (DBD-FISH) assays utilising like the comet assay agarose embedded cells but without applying an electrophoretic field (Fernandez et al. 2000; Fernandez et al. 2003). Other approaches like in situ nick translation (ISNT; Irvine et al. 2000) and terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL; Gorczyca et al. 1993) take advantage of enzymes, which are able to incorporate in situ marker-molecule-labelled deoxynucleotides onto the DNA to detect DNA damage very accurately. Another well-known, highly efficient assay, the SCSA, avails the metachromatic dye acridine orange and flow cytometry to assess the ratio of single-stranded to double-stranded DNA in a large number of individual sperm (Evenson et al. 1980; Love 2005). Despite the advantage of SCSA being a rapid, precise and objective measure of sperm DNA fragmentation (Evenson et al. 2007), the comet assay seems to be more selective, as it can detect various types of DNA damage, like DSB, SSB, ALS, and cross-links, and advantageously, only a few cells are needed for an exact evaluation (Wang et al. 2007). Nevertheless, the sperm comet assay and SCSA measure DNA damage by different principles, the conclusions arising from the data are similar (Morris 2002). Focusing on DNA integrity of male germ cells in general but on different endpoints like detection of nicks in situ (e.g. TUNEL), chromatin dispersion pattern (e.g. SCD) or the difference between single- and double-stranded DNA in sperm chromatin (e.g. SCSA), all these tests can perfectly corroborate findings evaluated with the comet assay.

Conclusion

In humans, more than 80% of all structural aberrations occur de novo and are of paternal origin (Thomas et al. 2006). Additionally to basic sperm damage (Trisini et al. 2004), the DNA from our reproductive cells may sustain even further damage from genotoxins due to lifestyle, environmental and medical exposure. Knowing this fact, it is of great importance to protect the integrity of our genome as effectively as possible. However, by only detecting major numerical and structural abnormalities, minor but potentially global chromosomal damage, which might play an important role in paternal genome abnormalities in miscarriage, is often underestimated (Virro et al. 2004). It is known that sperm DNA damage higher than 8% cannot be completely repaired in the oocyte and might lead to impaired embryo development and early pregnancy loss (Ahmadi and Ng 1999). It is therefore very important

to have a standardised assay in reproductive toxicology at hand, which can effectively target the male germ cells. To assess DNA damage per se in reproductive cells, the SCGE or comet assay has proven to be a reliable and rapid method (Fairbairn et al. 1995), hence being the most sensitive way to detect of DNA damage (Leroy et al. 1996). It is already used for regulatory purposes, e.g. UK Guidance on a strategy for testing of chemicals for mutagenicity (COM 2000). The importance of the sperm comet assay as a relatively new technique providing a sensitive assessment of genetic damage seems to have become widely recognised. This can be seen in the various publications available on background baseline and toxicology studies utilising male germ cells. In addition, changes and sophisticated modifications during the last few years have improved the potential of the comet assay even further, for example by introducing treatment with repair enzymes (Collins et al. 1993). Although guidelines are available for the assessment of DNA damage in the comet assay in somatic cells, unfortunately, no standardised comet protocol for sperm is available so far, which also accounts for a wide range of related procedures like sperm storage, which may lead to different baseline damage. It is therefore crucial for future reproductive toxicology studies on male germ cells to improve existing sperm DNA damage assessments and to develop more accurate diagnostic tests. At present, the comet assay with sperm, used in reproductive toxicology studies, seems to provide the necessary sensitivity, accuracy and flexibility for becoming a reliable test system for the biomonitoring of genotoxins and reprotoxins.

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