Mutation Research xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Mutation Research/Reviews in Mutation Research

journal homepage: www.elsevier.com/locate/reviewsmr
Community address: www.elsevier.com/locate/mutres

Review

The comet assay as an indicator test for germ cell genotoxicity

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ARTICLE INFO

Article history:
Received 7 September 2007
Received in revised form 19 March 2008
Accepted 20 March 2008
Available online xxx

Keywords:
Comet assay
DNA strand breaks
In vivo test
Genotoxicity testing
Gonads
Biomonitoring

ABSTRACT

The in vivo comet assay is a well-established genotoxicity test. It is currently mainly performed with somatic cells from different organs to detect a genotoxic activity of potential carcinogens. It is regarded as a useful test for follow-up testing of positive or equivocal in vitro test results and for the evaluation of local genotoxicity. However, the comet assay also has the potential to detect germ cell genotoxicity and may be used for demonstrating the ability of a substance or its metabolite(s) to directly interact with the genetic material of gonadal and/or germ cells. Such results are important for the classification of germ cell mutagens, e.g. in the context of the "Globally Harmonized System of Classification and Labelling of Chemicals" (GHS).

This review summarizes and discusses available information on the use of the comet assay with germ cells and cells from the gonads in genetic toxicology. The literature contains results from in vitro studies, ex vivo studies and in vivo studies. With regard to the assessment of germ cell genotoxicity, only in vivo studies are relevant but the other kind of studies provided important information on various aspects of the methodology. Many comet assay studies with human sperm have been performed in the context of male infertility and assisted fertilization. The results of these studies are not reviewed in detail here but various aspects of the assay modifications used are discussed. Measuring DNA effects by the comet assay in sperm requires additional steps for chromatin decondensation. Many different modifications of the alkaline and the neutral comet assay are in use but a standard protocol has not been established yet. High and variable background levels of DNA effects were reported and there is still need for standardization and validation of the comet assay with sperm. Some human biomonitoring studies with human sperm were published, but it seems to be premature to use these data for hazard identification and classification of chemicals. In contrast, the standard alkaline in vivo comet assay can easily be adapted to investigations with cells from reproductive organs. Tests with cells from the gonads (testis and ovary) seem to be most appropriate and a promising tool for demonstrating that a test compound reaches the gonads and is able to interact with the genetic material of germ cells. However, studies to standardize and validate these methods are necessary before the comet assay can be usefully applied in risk assessment of germ cell mutagens.

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doi:10.1016/j.mrrev.2008.03.005

Please cite this article in press as: G. Speit et al., The comet assay as an indicator test for germ cell genotoxicity, Mutat. Res.: Rev. Mutat. Res. (2008), doi:10.1016/j.mrrev.2008.03.005

1. Introduction

The comet assay (single-cell gel electrophoresis) is increasingly used in genotoxicity testing and human biomonitoring. It has gained popularity as a standard technique for evaluating DNA damage and/or repair due to its sensitivity for detecting low levels of DNA damage, its applicability to various tissues and/or special cell types, its requirement for small numbers of cells per sample and the general ease and speed of test performance [1,2].

In routine genotoxicity testing, the in vivo comet assay is performed to detect a genotoxic potential of industrial chemicals, biocides, agrochemicals, food additives and pharmaceuticals in various organs of experimental animals. There is general agreement that the alkaline version (pH > 13) is most appropriate because it detects a broad spectrum of primary DNA lesions (single- and double-stranded breaks, alkali labile sites, incomplete repair sites and crosslinks) with high sensitivity [1,3–5]. Acceptance of the in vivo comet assay by regulatory agencies is growing and this is mainly due to the fact that standard protocols have been developed by international comet assay workshops and the International Workshop on Genotoxicity Testing (IWGT) and further validation work is ongoing [3,5].

It is the aim of genotoxicity testing to assess the potential of chemical substances to be genotoxic carcinogens or to cause heritable damage in humans. Genotoxicity data are used in hazard assessment and in the classification/labelling of substances. In current test strategies, the in vivo comet assay is mainly performed with somatic cells from different organs to detect the genotoxic activity of potential carcinogens and is regarded as a useful test for follow-up testing of positive in vitro findings and for the evaluation of local genotoxicity [4].

However, the comet assay also has the potential to be a useful tool for investigating germ cell genotoxicity. The "Globally Harmonized System of Classification and Labelling of Chemicals (GHS)" has published classification criteria for germ cell mutagens, i.e. chemicals that may cause mutations in the germ cells of humans that can be transmitted to the progeny [6]. Category 1B defines "chemicals which should be regarded as if they induce heritable mutations in the germ cells of humans". Among the criteria that are given for this category, one requires "positive results(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has the potential to cause mutations in germ cells. This supporting evidence may, for example, be derived from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells". Although GHS does not explicitly mention the comet assay but only lists the sister chromatid exchange (SCE) analysis in spermatogonia and the unscheduled DNA synthesis test (UDS) in testicular cells as examples for genotoxicity tests in germ cells, the comet assay might play an important role in this context in the future. GHS requires that classification for heritable effects in human germ cells has to be made on the basis of well conducted, sufficiently validated tests, preferably as described in OECD test guidelines.

Although an OECD guideline for the comet assay is not available yet, recommendations for the performance of the comet assay were published by international expert groups. These recommendations follow current OECD guidelines for other in vivo genotoxicity tests and ensure that valid and reliable data are obtained [1,3,5]. However, these recommendations do not specifically consider the use of germ cells although it is obvious that the in vivo comet assay can be applied to germ cells and gonadal cells (i.e. spermatozoa, testis cells and ovarian cells). Tests with these cell types can be performed to determine germ cell genotoxicity and to demonstrate/exclude relevant exposure of germ cells or gonads to genotoxins, in particular to proven in vivo somatic cell mutagens. Various experimental studies using the comet assay with germ cells have been published but only limited data is available from routine testing in accordance with the above-mentioned recommendations and under conditions of "good laboratory practice" (GLP).

The comet assay is currently also frequently used in human biomonitoring for the detection of genotoxic effects in human populations exposed to genotoxins in the environment or at the workplace. The majority of human biomonitoring studies using the comet assay studied genotoxic effect in peripheral blood cells, used the alkaline version, and only a few of the investigations were performed with exfoliated epithelial cells [2]. Some studies have been published that evaluated genotoxic effects in human sperm after environmental exposure to chemicals. However, most of the comet assay studies with human sperm have been performed in the context of male infertility and assisted fertilization. Measuring DNA effects by the comet assay in sperm is a challenge because the highly organized and compact chromatin structure of sperm requires additional steps for chromatin decondensation. In these studies, various aspects of the methodology and factors that may influence the test results (e.g. the effect of cryopreservation of sperm samples, the use of antioxidants during cell preparation) have been addressed. In contrast to the studies with blood cells, various modifications of the comet assay with sperm (alkaline and neutral) are in use and there seems to be no general agreement up to now on the most appropriate approach. The publications in the context of the influence of sperm nuclear DNA integrity on assisted reproductive outcome will not be discussed in detail here but methodological aspects and data will be considered that are useful for a critical assessment of the results obtained with the comet assay with human sperm in biomonitoring.

It is the goal of this review to summarize and critically discuss available information on the use of the comet assay with germ cells and gonadal cells in genetic toxicology. The possibilities and limitations of measuring comet assay effects in human sperm will be discussed first. Then, data from experimental animal studies will be reviewed with regard to the sensitivity of the test and the reliability of the results. Finally, the use of the comet assay with gonadal cells in routine genotoxicity testing will be described and recommendations for future research will be given.

2. The comet assay with human sperm

DNA damage in human sperm has been investigated with the comet assay in the context of natural and assisted fertilization and in some biomonitoring studies measuring germ cell effects of exposure to environmental genotoxins/mutagens. It is striking that various comet assay modifications are in use including alkaline and neutral versions and applying different treatments for chromatin decondensation. It is the common goal of these approaches to enable DNA migration from the compact sperm chromatin while avoiding high background levels of DNA migration.

Sperm chromatin is a highly organized compact structure consisting of DNA and heterogeneous proteins. The condensed and insoluble nature of the sperm chromatin structure protects the genetic integrity during transport of the paternal genome through the male and female reproductive tracts. The sperm nuclear condensation process involves a complex sequence of events including topological rearrangements, transition of DNA binding proteins, loss of nucleosomal structure and formation of a chromosomal organization consisting almost entirely of highly condensed chromatin. Nucleoproteins exchange occurring in spermatids involves the replacement of somatic histones by

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transition proteins and the deposition of protamines that remain present in mature sperm. Sperm chromatin structure is being established during spermiogenesis and this process seems to involve the appearance of transient DNA strand breaks coincident with the chromatin remodelling steps [7]. The nature of the high background level of DNA strand breaks measured with the comet assay in ejaculated sperm is still unknown. It might be part of the normal differentiation program [7] but other mechanisms have been discussed such as aberrant chromatin packing during spermatogenesis, abortive apoptosis in the later stages of germ cell development or excessive production of reactive oxygen species [8].

To cope with these peculiarities of the sperm comet assay, various protocols have been established and are in use and manifold information is available on methodological and fundamental aspects that is helpful for a critical evaluation of the use of human sperm in genetic toxicology. The following aspects will be discussed:

(i) Comet assay modifications used for the detection of DNA effects in human sperm,
(ii) background levels of DNA effects in human sperm, and
(iii) ex vivo induction of DNA damage in human sperm.

2.1. Comet assay modifications used for the detection of DNA effects in human sperm

It was an early observation when the comet assay became established as a genotoxicity test that sperm exhibit increased background levels of DNA migration in comparison with somatic cells [9]. This effect was observed under denaturing alkaline conditions but did not occur when sperm DNA was electrophoresed at neutral pH nor under denaturing conditions not involving alkali. It was concluded that the increased migration observed for sperm cell DNA after alkali treatment is not due to preexisting DNA breaks but rather to the presence of alkali-sensitive sites [9]. Subsequently, the comet assay with sperm was frequently performed under neutral conditions. Since most of these protocols perform electrophoresis only under neutral conditions, authors often erroneously assume specificity for DNA double-strand breaks. It has been demonstrated, however, that such conditions just decrease the sensitivity of the assay while still mainly detecting DNA single-strand breaks [10,11]. Comet assay protocols for the detection of double-stranded breaks that actually reflect the behaviour of double-stranded pieces of DNA are available [12] but are rarely used. Therefore, the attempts to relate effects in the comet assay with sperm to single- or double-stranded DNA breaks is frequently an unjustified over-interpretation of the results.

While the standard (pH > 13) conditions were recommended by international expert groups for genotoxicity testing [1,3,5], there seems to be no generally accepted and recommended protocol for the comet assay with human sperm. Electrophoresis is carried out in different buffers at different pH values ranging from pH 8.2 to pH 13.5 [13–18]. As the extent of DNA migration in the comet assay is significantly influenced by the degree of alkali denaturation and the pH value used during electrophoresis, it is generally not possible to directly compare and evaluate the DNA effects reported in the different studies.

Another important aspect is the necessity to introduce additional steps (compared to the protocol with somatic cells) to decondense sperm chromatin and allow migration of DNA from the nucleus. Proteinase K is mainly used and added directly to the lysis solution [19] or proteinase K treatment is performed as an additional step after lysis [14]. Incubation with proteinase K is performed at room temperature, or at 37 °C and takes place for shorter (e.g. 3 h) or longer (e.g. overnight, 18 h) periods of time [14,18,20]. Other protocols include two enzyme digestion steps with RNase and proteinase K [14] and differ with regards to the addition of antioxidant (DMSO; dithiothreitol, DTT) to the lysis solution [15,20,21]. Another modification introduced incubation with DTT after lysis followed by incubation with lithium diiodosalicylate (LIS) [22]. Using this protocol, rather than overnight incubation with proteinase K, the total time taken for lysis and decondensation of DNA could be reduced to 3 h and the baseline DNA damage could be reduced [22]. Because the different approaches to decondense sperm chromatin may significantly influence the sensitivity of the comet assay and limit interlaboratory comparisons of results obtained with sperm under different test conditions, attempts to standardize and harmonize these protocols should be undertaken to establish the comet assay with sperm as a diagnostic tool.

2.2. Background levels of DNA effects in human sperm

Another limitation of the comet assay with human sperm as a test for detecting genotoxic effects becomes obvious when taking a critical look at the background frequencies of DNA effects reported in different studies. Table 1 summarizes some examples for

Table 1
Examples for baseline DNA migration in the comet assay with human sperm under different test conditions

Lysis/decondensation	Denaturation/electrophoresis	Effect	Reference
DMSO[a], proteinase K	Alkaline (pH > 13)	10–30% tail DNA[b]	Anderson et al. [25]
DMSO, proteinase K	Alkaline (pH 13)	~20% tail DNA[b]	Hughes et al. [15]
DMSO, proteinase K	Alkaline (pH 13)	15–60% tail DNA[b]	Hughes et al. [16]
DTT, proteinase K	Alkaline (pH 12.3)	97% tail DNA	Haines et al. [20]
DTT/LIS	Alkaline (pH 12.5/13?)	~10% tail DNA[b]	Donnelly et al. [22]
DMSO, proteinase K	Alkaline (pH 12.5)	6% "fragmentation"	Belcheva et al. [32]
DMSO, proteinase K	Alkaline (pH > 13)	~20% tail DNA[b]	Baumgartner [18]
DMSO, proteinase K	Alkaline (pH 13)	7.5% tail DNA	Migliore et al. [45]
DMSO, DTT	Alkaline (pH 13)	42% tail DNA	Schmid et al. [31]
DTT/LIS	Alkaline (pH 12.5/13?)	32% "fragmentation"	Agbaje et al. [17]
DMSO, DTT, proteinase K	PH 10	5% tail DNA	Bian et al. [40]
DTT; proteinase K	Neutral (pH 8.5)	33% tail DNA	Haines et al. [20]
DTT; proteinase K	Neutral (pH 8.2)	41% tail DNA	Morris et al. [13]
RNase, proteinase K	Neutral (pH 9)	20% tail DNA	Duty et al. [14]
RNase, proteinase K	Neutral pH 9	25–30% tail DNA	Hauser et al. [8]
DMSO, DTT	Neutral (pH 8)	35% tail DNA	Schmid et al. [31]

? indicates pH not exactly indicated.
[a] *Abbreviations*: DMSO, dimethyl sulfoxide; DDT, dithiothreitol; LIS, lithium diiodosalicylate.
[b] % head DNA was originally indicated and % tail DNA was calculated as 100 − % head DNA.

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baseline DNA migration from studies using different protocols for lysis/decondensation and different pH during denaturation and/or electrophoresis. It should be noted that DNA migration also depends on the duration of electrophoresis, the voltage and amperage. These conditions are fundamentally different in protocols with different pH and thus limit a direct comparison of results. Studies were selected which indicated the amount of DNA migration as "% tail DNA", "% head DNA" or "DNA fragmentation" to directly compare the effects. When % head DNA was originally indicated, % tail DNA was calculated (100 – % head DNA). Most of the published studies report background frequencies of DNA effects which are significantly higher than in somatic cells. The percentage of migrated DNA ranged from 5 to 97%. Although a direct comparison of an alkaline and a neutral version indicated lower background values in the neutral version [9,20,23] a comparison of publications from different groups (Table 1) shows that low levels of background damage can be obtained with alkaline versions as well. However, variability between studies seems to be higher with alkaline versions than with neutral versions. A protocol avoiding proteinase K treatment and using a combination of DTT and LIS led to low background frequencies (about 10% tail DNA) under alkaline electrophoresis conditions [22]. This modification might be promising not only because of the low background levels of DNA migration but also because of the short time period required for decondensation and denaturation of the DNA. However, a recent study of this group using the DTT/LIS decondensation with slight modifications reported 32% DNA fragmentation in sperm of healthy control subjects [17].

Taken together, these published studies suggest that the comet assay with sperm can be performed in alkaline and neutral versions. Specificity for DNA double-strand breaks was not demonstrated for the neutral versions and it has to be assumed that both methods mainly measure (directly and indirectly induced) DNA single-strand breaks. Background levels for DNA migration are rather high and variable for both protocols in most cases. However, assay modifications with acceptable levels of background damage seem to be available and, in combination with short time periods for processing the samples, they might enable a reliable detection of DNA effects in sperm with the comet assay.

2.3. Ex vivo induction of DNA damage in human sperm

Several comet assay studies tried to induced DNA effects in sperm ex vivo and thus characterized the sensitivity of sperm cells towards genotoxins in vitro. Some of these studies related such effects to the induction of DNA damage in somatic cells in vitro.

Studies with X-rays and hydrogen peroxide (H_2O_2) can be regarded as examples for the investigation of agents with clear genotoxicity and known mode of action. When human spermatozoa from fertile donors were treated with X-rays or H_2O_2 and analyzed by the alkaline comet assay, they seemed to be surprisingly resistant to damage compared to somatic cells [15]. X-ray irradiation up to 30 Gy did not lead to a significant induction of DNA migration and significant damage to control spermatozoa was induced only with 100 μM H_2O_2 which is about ten times higher than the effective concentration in somatic cells. With regard to X-irradiation, sperm seems to be more than 30-times less sensitive than somatic cells. The authors speculated that this insensitivity might result as a consequence of the marked condensation of the DNA which protects the DNA of mature spermatozoa [15]. In a later study of the same group, a DNA-damaging effect of X-ray irradiation at 10 and 30 Gy on human sperm was reported [21]. However, marginal effects were measured in comparison to unexposed controls and the statistical difference reported might be due to the use of each single cell as the experimental unit.

The relative insensitivity of the comet assay with human sperm to detect DNA damage induced ex vivo was also confirmed by Haines et al. [20]. Under neutral conditions 100 Gy gamma irradiation led to an increase in %tail DNA from 33.3 (controls) to 56.6. Interestingly, under alkaline conditions 97.4% of the DNA was already found in the tail and there was no further increase after irradiation with doses up to 100 Gy. Very similar results were obtained for mouse sperm [20]. A clear induction of DNA migration was found after exposure of sperm to 500 μM H_2O_2 in a study using proteinase K for decondensation [24] and with 200 μM H_2O_2 in a fourth study using the DTT and LIS modification of the assay [22].

Taken together, these publications suggest that despite a relatively high spontaneous DNA migration, genotoxic agents that induce strand breaks and oxidative DNA damage via the formation of free radicals do not readily lead to DNA effects in the comet assay with sperm under the same test conditions. The background effects clearly indicate that DNA strand breaks (or alterations leading to strand breaks under the conditions of the test) present in DNA of mature sperm actually lead to increased DNA migration. Consequently, the lack of DNA migration after irradiation or H_2O_2 treatment indicate that it is hard to induce damage in sperm with these agents, possibly due to the condensed nature of the chromatin and the lack of water necessary for ionization and various chemical reactions.

Only the group by Anderson and coworkers investigated the effects of several chemicals on DNA effects in the alkaline comet assay with sperm after ex vivo exposure. H_2O_2 was used as a reference substance but only led to an equivocal result in one study [25]. Concentrations up to 200 μM did not induce any effect in one experiment but a small decrease in % head DNA (median value 61.97 compared to 67.66 in the control) was measured in a second experiment with sperm from the same donor. The other compounds tested in this study (ß-estradiol, diethylstilbestrol, daidzein, genistein, nonylphenol, dibromochloropropane, ethylene glycol monoethyl ether, lead nitrate, 1,2-epoxybutene and 1,2,3,4-diepoxybutane) led to positive effects in the experiments (two or three) performed.

Further studies tested modulating effects of flavonoids on food mutagens in the comet assay with sperm [19,26]. 3-Amino-1-methyl-5*H*-pyrido (4,3-b)indole (Trp) and 2-amino-3-methylimidazo-4,5-f)quinoline (IQ) induced DNA effects in the comet assay with sperm. These effects were reduced in the presence of silymarin or myricetin, although these two compounds induced DNA effects in the comet assay with sperm when they were applied alone in the same range of concentrations. Very similar effects were observed in human lymphocytes and there was no fundamental difference between somatic and germ cells in the genotoxic as well as in antigenotoxic effects reported [26]. Furthermore, this group tested effects of hormones on DNA effects in human sperm [27,28]. Diethylstilbestrol, noradrenaline, triiodothyronine and L-thyroxine sodium salt slightly reduced the % head DNA in relation to a negative control in two experiments with a sperm samples from one donor. Parallel treatment of the sperm sample with catalase, and the flavonoids kaempferol and quercetin inhibited the hormone-induced DNA effects. The authors concluded that the hormone-induced DNA effects were caused by reactive oxygen species [28] although other studies (see above) suggested that it is hard to induce DNA effects in sperm with oxidative agents.

Finally, the well-known genotoxic anticancer drug doxorubicin was used to induce DNA damage in human sperm and lymphocytes in vitro [18]. Doxorubicin (formerly known as adriamycin) binds to

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DNA via intercalation, produces free radicals and stabilizes the topoisomerase II cleavage complex. It reduced the percentage of head DNA in sperm (0.8–1.6 μM) and in human lymphocytes (0.2–1.6 μM) but the effect in lymphocytes was not concentration-related. A similar decrease in % head DNA (or increase in tail moment) was observed for the whole range of concentrations tested. The similarity of the effects measured in the comet assay in lymphocytes and sperm should be interpreted with caution because the exposure protocols were rather different (sperm was treated for 1 h and analysed directly whereas lymphocytes were treated in cultures set up for chromosome preparations, cultivated for 20 h after treatment).

Taken together these studies indicate that the comet assay can be performed with sperm but the optimal conditions still have to be defined. In the context of genotoxicity testing, ex vivo experiments with sperm will not play an important role because genotoxic effects in sperm are only meaningful for hazard identification and risk estimation after in vivo exposure to mutagens. Only the in vivo approach allows us to consider the specific conditions of toxicokinetics, metabolism, damage persistence and repair that are relevant for the assessment of germ cell mutagenicity.

3. Human biomonitoring: detection of DNA damage in human sperm after in vivo exposure

The comet assay might be a useful tool for measuring genotoxic effects in sperm of humans exposed to environmental genotoxins. Results obtained with this assay might indicate a relevant exposure of human germ cells towards potential mutagens and might be useful for the evaluation of germ cell mutagenicity. Some human biomonitoring studies using the comet assay with sperm have been published, suggesting that genotoxic effects can be detected after various types of exposure. However, as already emphasized by GHS, data on humans in biomonitoring are variable and its use is limited [6]. Due to the known limitations of the techniques available, such studies have to be assessed on a case-by-case basis. Attention has to be paid to the adequacy of the exposure information and potential confounding factors.

With regard to confounding factors, three reports indicated an influence of age on comet assay effects in human sperm [29–31]. Using a neutral version of the comet assay, it was shown for a group of 66 men between the ages of 20 and 57 years that age correlated with an increasing percentage of sperm with enhanced DNA damage (range: 0–83%) [29]. Trisini and co-workers found that men older than 35 years had a statistically significant increase in the number of cells with high DNA damage in the neutral comet assay as compared with younger man [30]. A recent study reported increased sperm DNA damage in older men in the alkaline comet assay but not in the neutral version [31]. Surprisingly, this group also showed an effect of substantial daily caffeine consumption in the neutral version but not in the alkaline version of the comet assay with sperm.

Cigarette smoking is another potential confounding factor in human biomonitoring for genotoxic effects. A comparison between the DNA effects in smokers ($n = 25$) and non-smokers ($n = 15$) revealed that DNA damage in spermatozoa of smokers is increased in the comet assay (pH 12.5) but the difference to non-smokers (percentage DNA fragmentation 7.4 vs. 5.9) was not statistically significant [32]. Considering the fact that the comet assay does not unambiguously detect genotoxic effects of smoking in peripheral blood of smokers [33], it has to be questioned whether a clear effect in sperm can be expected at all.

Of crucial importance for the reliability of the sperm comet assay in biomonitoring are stable background frequencies in unexposed subjects (negative control) and clear and reproducible effects of known in vivo genotoxins (positive control). However, as already discussed (see Section 2.2) background levels are variable and strongly depend on the comet assay modification used. Furthermore, it is still unclear whether these baseline levels represent real background levels of DNA damage, other physiological effects of sperm development and chromatin structure or just artefacts produced by the decondensation and denaturation steps of the methodology.

Study groups with clear and defined mutagen exposure which are suited as a positive control are rare in biomonitoring. One exception are cancer patients after chemotherapy with DNA-damaging compounds. Only one study is available that tested DNA damage in sperm of one patient with chronic lymphocytic leukaemia after therapy with fludarabine [34]. An increase in DNA migration in a neutral comet assay was measured 8 weeks but not 4 weeks after the start of the chemotherapy. However, this effect was only measured once in one patient and it has to be questioned whether it is treatment-related at all. Fludarabine is a purine analog that inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase. It does not directly induce DNA damage. DNA effects in the comet assay may occur during disturbed replication but such an effect does not persist and should not be present in mature sperm. Thus, exposure to fludarabine is not an optimal choice for a positive control and other relevant exposures (e.g. alkylating compounds) have not been tested yet. Such studies should be performed to prove that the comet assay with sperm is actually able to detect DNA-damaging effects after relevant and defined in vivo exposure to known mutagens.

One group used the comet assay to study associations between urinary levels of environmental xenobiotics and DNA effects in sperm [8,35–37]. A neutral version (pH 9) of the comet assay with RNase and proteinase K treatment for decondensation of the chromatin was used in these studies. No association between serum levels of some chlorinated organic compounds and DNA effects in the comet assay with sperm was determined in a study with 212 male partners of sub-fertile couples [35]. Positive effects in the comet assay with human sperm have been reported after exposure to the insecticides carbaryl and chlorpyrifos [37] and to phthalates [8,36]. For 260 subjects of the general population exposed to the non-persistent insecticides carbaryl and chlorpyrifos at low levels an association between urinary metabolite concentrations and comet assay effects was calculated [37]. No consistent association between effects in the comet assay and urinary insecticide metabolite concentrations were found. A strong association was determined for % tail DNA but there was a negative correlation between metabolite levels and comet lengths. The most likely explanation is that the observed effects are actually unrelated to the assumed exposure. An in vivo comet assay with leukocytes of mice after oral exposure to chlorpyriphos (up to 9 mg/kg body weight) revealed a positive result [38]. However, the effects were small, were measured 24 h after acute exposure and did not persist. 96 h after exposure tail lengths had reached control levels again. Considering this result one would not expect effects in the in vivo comet assay after normal environmental exposure conditions. An association was also reported for urinary levels of some phthalates at environmental levels (i.e. in a study population without identified sources of exposure to phthalates) with comet assay parameters [8,36]. This association is critically discussed by the authors because phthalates do not possess a clear genotoxic potential in vivo. Therefore, a genotoxic effect in the comet assay with sperm is unexpected and might represent a chance finding. Using another test for sperm integrity (the sperm chromatin structure assay) a study among Swedish men did not find an

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association between urinary phthalate metabolites and DNA damage [39].

Taken together, these studies do not demonstrate a causal relationship between urinary levels of environmental chemicals and DNA damage in sperm and it seems to be unlikely that the comet assay with human sperm is suited to detect genotoxic effects of compounds with low genotoxic potential at usual environmental exposure levels.

Recently, occupational exposure to the pesticide fenvalerate (FE) was reported to be associated with an increase in sperm DNA damage [40]. The comet assay was performed at pH 10 and slides were incubated in a DTT solution after lysis and then in a solution containing proteinase K. A slight but statistically significant increase in % tail DNA from 5.6 in the control group ($n = 23$) to 11.3 in the exposed group was found. FE is supposed to be a hormonally active compound and associated with testicular toxicity. It induced genotoxicity/clastogenicity in cultured human blood cultures in vitro at high (toxic) concentrations [41,42] but data on its in vivo mutagenicity are not available. It is unclear at present whether the observed comet assay effects in sperm are actually related to induced DNA damage, or a consequence of indirect effects of incomplete maturation during spermiogenesis – as speculated by the authors – or just a chance finding.

Sperm samples from 30 workers occupationally exposed to acrylonitrile for 2.8 years was investigated in comparison to 30 controls [43]. A pH 10 version of the comet assay using DTT, RNase and proteinase K for chromatin decondensation was performed. The mean comet tail length was 9.8 in the exposed group versus 4.3 in the control group ($p < 0.01$). An study performed within the National Toxicology Program (NTP) [44] reported that acrylonitrile was genotoxic in vitro but not in vivo (it did not induce micronuclei in peripheral blood of mice after exposure by gavage for 14 weeks). A genotoxic effect in human sperm as a consequence of induced DNA damage seems to be unlikely but the actual cause for the effect seen in the study by Xu et al. [43] is not clear.

Sperm samples from 42 male subjects exposed to styrene and 25 controls were analysed for DNA effects by the comet assay with sperm [45]. Workers from factories manufacturing glass–fibre reinforced plastics and boats were exposed to styrene at least for 2 years in the past 5 years and continuously for 6 months before sampling. Urinary excretion of the major styrene metabolites, mandelic and phenylglyoxylic acids, indicated on the group level that styrene exposure was above 20 ppm. DNA damage in sperm was determined by the alkaline (pH 13) comet assay with proteinase K treatment as established by McKelvey-Martin et al. [46]. A statistically significant difference between styrene exposed and control subjects (11.02% vs. 7.42% tail DNA) was measured ($p < 0.001$; Student's t-test). This result confirmed an earlier study from the same group reporting results from 46 exposed male workers and 27 controls [47]. Exposure data (mean period of employment 9.2 years) and the results of the comet assay were nearly identical (10.9% vs. 7.4% tail DNA) and it can be assumed that these two studies are not completely independent. Induction of DNA migration in the comet assay in somatic cells (peripheral blood) was reported for workers exposed to styrene in several studies (reviewed by Henderson and Speit [48]). However, the results were not consistent and the positive effects were small and not consistently related to exposure levels. A recent review of the in vivo genotoxicity tests performed with styrene came to the conclusion that there is no clear evidence that styrene induces mutagenic/clastogenic effects in vivo when tested under appropriate test conditions [48]. Although induction of DNA strand breaks and oxidative base damage in mice exposed to styrene by inhalation was reported [49], a critical evaluation of the findings revealed that they were equivocal [50]. In the absence of a clear mutagenic potential of styrene in vivo, genotoxic effects in sperm are not plausible and have to be assessed with caution considering the limitations of the assay.

In summary, comet assay studies with sperm in human biomonitoring are still in an experimental state and the results obtained so far should be interpreted with caution. Further investigations are needed to clarify the biological significance of the background levels and to unequivocally demonstrate comet assay effects in sperm after defined in vivo exposure to known mutagens. Due to the uncertainties of the methodology positive findings in biomonitoring studies associated with exposure to mutagens are not suited to prove relevant exposure of germ cells or indicate a genetic risk. At present, such studies do not have the necessary degree of reliability to be useful for risk assessment or the classification of mutagenic compounds.

4. The comet assay with germ cells and gonadal cells of experimental animals

Manifold data is available in scientific publications on DNA effects in the comet assay with germ cells from experimental animals. Effects were measured in sperm or in testis cells after exposure of the animals to ionizing radiation or various chemicals using different modifications of the comet assay.

Spermatozoa from the vas deferens of mice were investigated 45 days after irradiating the testis with X-rays (4 Gy) or the isotope Indium-114 m (1.85 MBq). An alkaline version (pH 8) of the comet assay was performed using DDT in the lysis buffer and proteinase K treatment for decondensation [51]. Significantly increased DNA migration was measured after both types of irradiation in comparison to unexposed controls. This results suggests that DNA damage arising in spermatogenic cells can be detected 45 days later in resultant sperm. The levels of DNA effects in spermatozoa were similar after both internal and external irradiation in spite of the accumulated testicular dose from Indium-114 m being over twice that of the X-rays. It is unclear how DNA damage induced in spermatogonial cells could remain through several rounds of mitosis and a successful meiosis and be found as strand breaks in mature spermatozoa. However, increased DNA effects in murine spermatozoa after testicular X-irradiation measured by the neutral comet assay were confirmed in a second study [52]. A dose-related effect was demonstrated in the dose range 0.5 to 4 Gy 45 days after irradiation. A time-dependent increase in DNA effects was measured 16, 31 and 45 days after irradiation while after 120 days the effect decreased to the level measured at day 16. The authors conclude from these data that the maximum effect represents induction of damage in differentiating spermatogonia but also that damage induced in stem cell spermatogonia seems to persist [52]. Similar results were reported in an independently performed study after irradiation of murine testis with 1–4 Gy and measuring effects in the comet assay after 14, 45 and 100 days [23]. A dose-related effect was measured in testis cells with the alkaline (pH 13) comet assay 14 days after irradiation. The analysis of different cell subpopulations revealed that increased DNA effects were detected only in the tetraploid cell compartment which is composed mainly of primary spermatocytes actively engaged in the meiotic process. The comet assay with spermatozoa was performed under neutral (pH 8) and alkaline (pH 12.1) conditions using DTT in the lysis solution for chromatin decondensation. An increase in DNA strand breaks was measured in both versions 45 days after exposure, while there was no significant effect after 14 or 100 days. The level of damage appeared to be higher in spermatozoa than in primary spermatocytes and the authors suggest that the DNA effects in mature

Please cite this article in press as: G. Speit et al., The comet assay as an indicator test for germ cell genotoxicity, Mutat. Res.: Rev. Mutat. Res. (2008), doi:10.1016/j.mrrev.2008.03.005

sperm derive from an active process of DNA fragmentation occurring during meiosis and subsequent spermiogenesis [23].

Cells from the testis of mice exposed to whole body irradiation by X-rays were investigated in the alkaline (pH > 13) standard comet assay 24 h after exposure. A significant increase in DNA migration was determined for the dose range 0.5–2 Gy. The dose-response was similar to the effects measured in bone marrow of the same animals at the same time point [53].

Cell phase specificity of DNA effects induced by cyclophosphamide (CP) were studied in cauda epididymal spermatozoa with the alkaline (pH 12.1) comet assay using a protocol with DMSO, DTT and proteinase K for chromatin decondensation [54]. Adult male rats were treated with CP according to four different protocols: (1) once with 100 mg/kg, (2) four times daily with 6 mg/kg, (3) four times daily with 100 mg/kg at day 1 and 50 mg/kg at days 2–4, and (4) 6 mg/kg daily for 14–28 days. To determine the effect of CP during late, mid- and early spermiogenesis, animals were sacrificed on days 14, 21 and 28, respectively. Acute exposure (protocol 1) led to slightly increased DNA effects on day 14, equivocal effects on day 21 and no significant effect on day 28. Low dose sub-chronic exposure (protocol 2) slightly increased DNA migration on day 21 and this effect was more pronounced after sub-chronic exposure to the higher doses (protocol 3). Increased DNA effects were measured after chronic exposure to 6 mg/kg CP (protocol 4) at all three-time points investigated. The increase in damage appeared to reach a plateau by day 21, as there was no difference between the fold increases in damage for days 21 and 28. The authors conclude that spermatids during mid-spermiogenesis show increased susceptibility towards CP-induced DNA damage. As the magnitude of DNA damage at all time points after low-dose chronic exposure was much greater than that following low-dose exposure for 4 days, damage seemed to accumulate over time [54].

A chemotherapy cocktail used to treat testicular cancer containing bleomycin, etoposide and cisplatinum (BEP) was tested for its effects on sperm chromatin integrity in rats after treatment for 3, 6 and 9 weeks. DNA damage in spermatozoa was measured by the comet assay only after 9 weeks of treatment with BEP doses adjusted for surface area and equivalent to 1/3×, 2/3× and 1× of the human dose. A pH 10 modification of the comet assay was performed with DMSO and DTT in the lysis solution and treatment with proteinase K for chromatin decondensation [55]. A significant increase in DNA migration was only found when the rats were treated with the highest dose (equivalent to the human therapeutic dose).

A single intraperitoneal injection of cyclophosphamide (CP) or mitomycin C (MMC) induced dose-related DNA effects in the standard alkaline (pH > 13) comet assay with testis cells of mice 24 h after treatment [53]. Significant differences in comparison to negative controls were obtained for 3.15 mg/kg CP and for 0.25 mg/kg MMC. Testis cells were slightly more sensitive towards the DNA-damaging effects of these two antineoplasic drugs than bone marrow cells which were investigate in parallel. It is unclear, why the potent DNA–DNA crosslinker MMC caused increased DNA migration instead of the expected reducing effect [56].

Surprisingly, the same author reported positive effects in the comet assay with testis cells of mice 35 days after a single intraperitoneal injection of vincristine (VCR, 1 or 2 mg/kg) [57]. This result is unexpected because VCR does not interact with DNA and does not produce persistent DNA damage. It is therefore rather unlikely that the comet assay results represent induced DNA damage but seem to be due to a secondary and unspecific effect.

Vanadium pentoxide was tested for its ability to induce DNA damage in testis cells of mice 24 h after a single intraperitoneal injection [58]. A standard alkaline (pH 13) comet assay was performed and a significant induction in DNA migration was measured for 5.75, 11.5 and 23.0 μg/g vanadium pentoxide. In contrast, sodium ortho-vanadate (NA_3VO_4) did not induce DNA effects in testis cells of mice treated for 5 weeks with NA_3VO_4 in drinking water up to a dose of 1500 mg/l [59]. Interestingly, this treatment led to increased frequencies of micronuclei but not to increased comet assay effects in bone marrow cells. DNA migration was only enhanced in splenocytes of mice at the highest dose.

1,3-Butadiene (BD) and its metabolites, 1,2-epoybutene and 1,2,3,4-diepoxybutane were tested in the standard alkaline comet assay with testicular cells [60]. Exposure of mice to BD by inhalation for 4 weeks (6 h/day) did not induce DNA effects at dose levels up to 130 ppm. Negative results were also reported for liver and bone marrow. Treatment with 1,2-epoxybutene (intraperitoneal injection) induced DNA migration in testes of mice (dose: 120 mg/kg) but not in rats. By contrast, this treatment led to increased DNA migration in bone marrow cells of rats but not of mice. An intraperitoneal injection of 1,2,3,4-diepoxybutane did not induce effects in the comet assay with testicular cells of mice (15 or 30 mg/kg) or rats (12.5, 25 or 50 mg/kg). In a second study of this group, male mice were exposed to 1,3-butadiene (BD) by inhalation for a single 6 h period or for 10 weeks (6 h/day, 5 days/week) and DNA damage was measured by the alkaline comet assay in testicular cells [61]. After acute treatment, exposure to 125 ppm BD resulted in a modest but statistically significant increase in DNA damage both in haploid and polyploidy cells of the testis. No DNA-damaging effect was apparent after 10 weeks (inhalation) treatment with 12.5 or 125 ppm BD.

In summary, these studies show that the comet assay with sperm after in vivo exposure of experimental animals to mutagenic chemotherapeutic drugs (CP, BEP) for longer time periods (2–9 weeks) reveals increased DNA damage. Further studies are necessary to characterize the sensitivity and reproducibility of this approach. It should be demonstrated that comet assay effects in sperm are causally related to DNA damage and do not represent unspecific effects as some results (e.g. vincristine) suggest. Testis cells do not seem to differ much from somatic cells with regard to the sensitivity towards irradiation and chemical mutagens and might be appropriate for in vivo genotoxicity testing of potential germ cell effects.

5. The in vivo comet assay with gonadal cells as a routine test in genotoxicity testing

In our experience, it is possible to adapt the standard alkaline comet assay to investigations with cells from reproductive organs. Besides cells from the testis and the ovary, other organs of interest can be studied such as the prostate, uterus and mammary gland. The comet assay may also be used to detect genotoxicity in organs within the neuroendocrine pathway (e.g. pituitary, hypothalamus cells) that control secretion and regulation of gonadotropic hormones potentially involved in carcinogenesis in reproductive organs. For demonstrating the ability of a substance or its metabolite(s) to interact with the genetic material of germ cells, the investigation of cells from the gonads (testis and ovary) seem to be most appropriate. Although different types of cells (including many somatic cells) are present in these organs and the comet assay does usually not discriminate between different cell types, a positive effect in the comet assay with cells from gonads indicates relevant exposure of the gonad. Table 2 summarizes results from in vivo comet assays with Sprague Dawley rats dosed with a single oral administration of 300 mg/kg EMS in comparison with the negative control (dH_2O). Data for the liver are included for a comparison with a standard tissue for somatic cell effects. Baseline and EMS-induced DNA effects are also shown for other reference

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Table 2
Baseline and EMS-induced DNA effects in the in vivo comet assay with gonadal and reference tissues

Study	Tissue	Sex[a]	Treatment	Tail DNA (%)	OTM
1	Liver	M	Control0	6.6 ± 0.52	0.7 ± 0.09
1	Liver	M	EMS	26.7 ± 0.69	6.7 ± 0.32
2	Liver	M	Control	5.8 ± 0.14	0.8 ± 0.06
2	Liver	M	EMS	21.2 ± 0.96	5.1 ± 0.28
3	Liver	F	Control	7.2 ± 0.47	1.2 ± 0.10
3	Liver	F	EMS	35.9 ± 2.03	11.2 ± 0.82
5	Liver	F	Control	8.5 ± 0.99	1.5 ± 0.36
5	Liver	F	EMS	29.8 ± 1.52	8.1 ± 0.61
1	Testis	M	Control	8.9 ± 0.58	1.2 ± 0.19
1	Testis	M	EMS	20.6 ± 2.00	3.5 ± 0.51
2	Testis	M	Control	8.1 ± 0.56	1.0 ± 0.11
2	Testis	M	EMS	20.6 ± 0.63	4.0 ± 0.27
3	Ovary	F	Control	10.3 ± 0.59	1.7 ± 0.18
3	Ovary	F	EMS	42.3 ± 2.47	11.7 ± 0.84
5	Ovary	F	Control	17.4 ± 1.40	4.3 ± 0.56
5	Ovary	F	EMS	28.8 ± 0.90	8.6 ± 0.50
3	Uterus	F	Control	29.8 ± 1.21	9.8 ± 0.48
3	Uterus	F	EMS	45.8 ± 1.62	16.3 ± 0.84
5	Uterus	F	Control	28.8 ± 1.74	9.2 ± 0.79
5	Uterus	F	EMS	35.9 ± 0.60	11.6 ± 0.37
3	Mammary Gland	F	Control	12.5 ± 1.62	2.7 ± 0.49
3	Mammary Gland	F	EMS	35.2 ± 2.36	9.7 ± 0.88
5	Mammary Gland	F	Control	15.1 ± 1.38	3.8 ± 0.57
5	Mammary Gland	F	EMS	29.4 ± 1.52	8.2 ± 0.69
1	Prostate	M	Control	13.3 ± 0.92	2.7 ± 0.28
1	Prostate	M	EMS	27.1 ± 1.11	7.6 ± 0.34
2	Prostate	M	Control	14.5 ± 1.95	3.5 ± 0.70
2	Prostate	M	EMS	31.7 ± 2.53	8.8 ± 0.96

Abbreviations: PK, proteinase K; EMS, ethyl methanesulfonate (300 mg/kg); M, male; F, female; OTM, Olive tail moment.
[a] Data represent mean values from six animals per group.

tissues that might be useful for the detection of gender-specific effects (uterus, mammary gland, prostate). Single-cell suspensions were prepared from the different tissues by mincing the tissues with fine scissors in cold mincing solution. Testis cells were isolated from a portion of testis but also included the head of the epididymis. Ovary cells represent a portion of an ovary including a section of the connected fallopian tube. The central portion of the uterine body including a portion of the cervix was used to study uterus cells and from the prostate a portion of the ventral lobe was prepared. The comet assay was performed at Helix3 Inc. according to a slightly modified standard protocol [62] with lysis overnight, alkaline unwinding (pH > 13) for 20 min and electrophoresis (0.7 V/cm) for 40 min. Slides were stained with SYBR Gold™ stain and 100 cells per sample (50 cells per replicate slide) were scored using image analysis (Komet GLP Image Analysis System; Kinetic Imaging Ltd.). Two measures for DNA migration are shown, % migrated DNA (% tail DNA) and Olive tail moment (OTM).

It can be seen (Table 2) that the background effects in testis and ovary cells are in a normal range in comparison with liver under these experimental conditions. EMS clearly induced DNA migration in cells from male and female gonads. Although the results clearly indicate that EMS can reach the gonads in only 4 h after a single oral exposure, this protocol may not be appropriate for testing all compounds for germ cell genotoxicity with the comet assay. The appropriate exposure conditions and/or sample time(s) for testing unknown compounds should be determined prior to the performance of the comet assay based on pharmacokinetic data if possible to ensure that gonadal exposure to the compound would be achieved at the time of sampling. It is especially critical for negative comet assay results to scientifically justify the experimental conditions used or to provide adequate evidence of relevant gonadal exposure by other means. As in all comet assay studies, the evaluation of cytotoxicity in the target organs should also be included in the interpretation of the comet assay results with gonadal cells.

Although the results shown here suggest that the comet assay in gonadal cells may be included in routine genotoxicity testing for germ cell genotoxicity, there is still need for further validation. Comparative investigations with different types of chemicals and different treatment protocols (single vs. repeated treatment) should be performed in different laboratories under standardized conditions.

6. Conclusions

The comet assay with sperm has been frequently used in the context of natural and assisted fertilization and male infertility. Many different comet assay modifications are in use including alkaline and neutral versions. Attempts to standardize and harmonize these protocols should be undertaken to enable a direct comparison of results and to establish the comet assay as an accepted diagnostic tool.

Human spermatozoa from fertile donors seem to be surprisingly resistant to the ex vivo induction of DNA damage by know genotoxins such as H_2O_2 and ionizing radiation compared to somatic cells but other studies reported induction of DNA migration by weak genotoxins and substances which do not directly interact with DNA. It might be useful for a better

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understanding of this approach to establish concentration–effect relationships for a series of standard mutagens with different modes of action. However, there is no need for an ex-vivo assay with sperm in genetic toxicology because in vivo exposure is needed to assess germ cell genotoxicity.

Limited data is available from biomonitoring studies with sperm from human subjects exposed to environmental mutagens. However, this approach is still in an experimental state and the sensitivity and reproducibility of this in vivo genotoxicity test needs to be determined. At present, it seems to be premature to consider such studies for risk assessment or the classification of mutagenic compounds.

The in vivo comet assay with gonadal cells from experimental animals might be a promising test for germ cell genotoxicity. However, a standard protocol (following the guidelines for the in vivo comet assay with somatic tissues) including appropriate exposure schedules and sampling times needs to be established and validated first.

Acknowledgements

The work of one of the authors (GS) is financially supported by the Styrenics Steering Committee (SSC). The interpretation of the data presented in this review, as well as the conclusions reached herein, are the sole responsibility of the authors.

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