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Review

Application of the comet assay for the evaluation of DNA damage in mature sperm

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ABSTRACT

DNA integrity is considered an important parameter of semen quality and is of significant value as a predictor of male fertility. Currently, there are several methods that can assess sperm DNA integrity. One such assay is the comet assay, or single-cell gel electrophoresis, which is a simple, sensitive, reliable, quick and low-cost technique that is used for measuring DNA strand breaks and repair at the level of individual cells. Although the comet assay is usually performed with somatic cells from different organs, the assay has the ability to detect genotoxicity in germ cells at different stages of spermatogenesis. Since the ability of sperm to remove DNA damage differs between the stages, interpretation of the results is dependent on the cells used. In this paper we give an overview on the use and applications of the comet assay on mature sperm and its ability to detect sperm DNA damage in both animals and humans. Overall, it can be concluded that the presence in sperm of significantly damaged DNA, assessed by the comet assay, is related to male infertility and seems to reduce live births. Although there is some evidence that sperm DNA damage also has a long-term impact on offspring's health, this aspect of DNA damage in sperm is understudied and deserves further attention. In summary, the comet assay can be applied as a useful tool to study effects of genotoxic exposures on sperm DNA integrity in animals and humans.

1. Introduction

The comet assay (single-cell gel electrophoresis) is a relatively simple, sensitive, reliable, fast and low-cost technique that is generally used to measure single- and double-strand DNA breaks. Modifications of the standard protocol also enable the detection of specific types of DNA base modifications, and the repair of such damage at the level of individual cells. The comet assay is commonly applied in basic research, genetic toxicology, regulatory toxicology, risk assessment, and human biomonitoring [1–12]. Essentially, cells are embedded as a single cell suspension in an agarose matrix, followed by lysis with high salt and detergent, leaving DNA attached to the nuclear matrix as nucleoids. Subsequently, nucleoids embedded in gels are subjected to electrophoresis. After neutralization, staining with a fluorescent dye and visualisation by fluorescence microscopy, quantitative analysis based on the distribution of the fluorescence pattern in each nucleoid is used in order to determine the extent of DNA damage induced, indicated by the relative intensity of the 'comet tail' [1,2,5,12,13].

The comet assay was first introduced by Östling and Johanson in 1984 [14] and four years afterwards its more used alkaline version was developed by Singh and colleagues in 1988 [15]. In the years that followed, the assay was adapted and modified for the measurement of a whole range of different types of DNA damages. Digestion of DNA with a lesion-specific repair enzyme allows measurement of a range of altered bases. The most popular enzymes currently used are endonuclease III (EndoIII), formamidopyrimidine-DNA glycosylase (Fpg), and 8-oxoguanine DNA glycosylase 1 (OGG1), which catalyse the excision of various forms of DNA damage arising from oxidative insults [16–18]. These

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modified protocols for the measurements of DNA oxidation damage are very popular and useful in terms of exploring mechanisms behind induced DNA damage. One of the newest modifications of the comet assay can be used to evaluate epigenetic changes [19–22]. It is also possible to measure other DNA lesions such as DNA crosslinks [23,24]. Using such modifications provides more specific understanding of the type of DNA damage induced. Detailed protocols for the comet assay can be found in the following papers [1,5,12,25,26] including Minimum Information for Reporting Comet Assay (MIRCA) procedures and results [27]. Here, we will briefly describe a protocol for the sperm comet assay (Box 1).

The generally accepted explanation of comet formation is that lysis removes histones but the DNA remains attached at intervals to the nuclear matrix, and in a supercoiled state. The presence of DNA breaks relaxes supercoiling and allows extension of DNA loops during electrophoresis towards the anode, creating the 'comet tail' [5]. However, in sperm, the DNA is differently packed, because histones are replaced by protamines [28,29]. Therefore, the standard protocol of the comet assay needs to be adapted when it is applied to sperm.

An additional challenge when using sperm is the timing of sampling; in humans usually cells from an ejaculate are used, which represents mature spermatids, but in animal studies other stages of sperm development can be collected and tested, including spermatocytes and spermatogonia. In these latter cells, DNA is not yet densely packed and transient strand breaks may be present because of active recombination processes [30]. Moreover, earlier developmental stages of sperm still have active DNA repair, whereas mature sperm from an ejaculate lack active repair activity [31]. These aspects make it more difficult to correctly interpret sperm comet data, and therefore in this paper we focus on DNA damage in mature sperm.

In this integrative review, we give an overview of the use and application of the comet assay on sperm, especially in reproductive toxicology and human reproduction. We conducted a search to identify relevant papers using scientific databases, including PubMed (www.pubmed.com), Web of Knowledge (www.webofknowledge.com) and Scopus (www.scopus.com). The titles and abstracts were assessed to consider the articles for inclusion in the review. We did not apply any restriction concerning publication language, country or the dates of publication. Other relevant original and review papers were also identified from the reference lists of papers found in the search and those papers are included in the present review.

2. Origin of sperm DNA damage

Today, we are exposed to a variety of potentially harmful chemical and physical agents in our daily life from environmental and occupational settings, as well as from different diagnostic and medical treatments. Many of these compounds can pass the blood testes barrier. Therefore, our reproductive cells are of particular concern as they pass on our genetic material to the next generation. Sperm DNA breakage may represent a threat to male fertility, human reproduction and the health of the offspring. The causes of sperm DNA damage are still unclear although it has been hypothesised that the damage may arise due to a phenomenon called aborted apoptosis in which unfunctional Fas signalling can allow the apoptotic cells to escape apoptotic cell death [32–35]; however, it is questioned whether apoptosis related breaks can be seen in the comet assay at these low size levels. Besides, oxidative insults as well as defects in chromatin maturation due to protamination failures are other causes of sperm DNA damage [36–41]. Additionally, the enzyme topoisomerase can create DNA breaks during the protamination process and if these breaks are not repaired efficiently, they can result in permanent DNA damage in mature sperm [30,42,43]. The above-mentioned mechanisms are interlinked since sperm with defective chromatin compaction are more susceptible to DNA oxidative damage, which in turn can trigger apoptotic cell death [32,44,45].

It has been assumed for a long time that defects in the genetic material of the paternal germ line can influence the course of embryonic development, following the first observations on the relationship between birth order and the incidence of achondroplasia by Weinberg in 1912 [44,46,47]. Nevertheless, the potential significance of DNA damage in mature spermatozoa was acknowledged only after the study done by Singh et al. [48] showing the presence of large numbers of single-strand DNA breaks in sperm using the alkaline comet assay. That study detected around 10^6 to 10^7 breaks per genome in human and mouse spermatozoa, but the same level was not found in human lymphocytes or in mouse bone marrow cells. Later, it was concluded that such breaks might be physiological and related to the compaction of the entire haploid genome into just 5 μm^3, the size of a human sperm head [44,49].

The observed higher frequency of DNA damage in sperm compared to somatic cells may be due to the susceptibility of sperm to damage during different stages of their development, inside the testes, epididymis and/or ejaculate but may also depend on the differences in

Box 1
Sperm comet assay protocol.

- Cells are embedded in agarose matrix on a glass or plastic support.
- After solidification, samples are immersed in a freshly prepared lysis solution (in order to remove membranes and soluble components) supplemented with dithiothreitol (DTT; in order to break disulphide bonds in protamines) and proteinase K (in order to remove protamines that could impede DNA migration through the agarose).
- Samples are then washed with deionized water in order to remove salt and detergent from the micro-gels.
- The gel samples on their plastic or glass support are placed on a horizontal gel electrophoresis tank, facing the anode. The unit is filled with fresh denaturation buffer and the samples are placed in it to allow DNA denaturation and unwinding.
- Subsequently, electrophoresis is carried out at a specific electric voltage (V/cm) in the same buffer under dim light to allow DNA migration.
- Afterwards, samples are rinsed gently with deionized water to remove excess alkali and detergents in order to neutralize them.
- Samples are stained with fluorescent dye, and subsequently analysed.
- A total of 100 (minimum of 50) randomly captured comets from each gel sample should be analysed using an epifluorescence microscope connected by camera to a commercially available image analysis system.
- A computerized image analysis system is used to obtain images, compute the integrated intensity profile for each nucleoid, estimate the comet components and evaluate the range of derived descriptors (% DNA in tail, tail length and/or tail moment).
- Percentage tail DNA (tail intensity) should be used as the recommended comet assay descriptor. The mean or the median of the scored comets is used to describe each sample.

protocol. Another issue is that sperm, when differentiated, cannot repair the DNA damage or undergo apoptosis, even when they are highly damaged [31,33,40,50,51] since the cellular machinery that allows male germ cells to complete repair or apoptosis is discarded during spermatogenesis. DNA damage in sperm may also arise from incomplete rejoining of the nicks within the DNA produced during the remodelling of the sperm chromatin. Damage may also be done to mature sperm by ROS-producing immature sperm during co-migration through the epididymis or by ROS-producing epithelial epididymal cells. Moreover, sperm DNA damage may also occur through the mechanism of sperm DNA fragmentation (SDF) [32,52,53]. It is worth pointing out that oxidative stress is regarded as a major cause of sperm DNA damage. Sperm are susceptible to ROS-induced damage because they have a high content of polyunsaturated fatty acids and no ability to repair this type of damage [40,50,54–59].

Sperm DNA damage can be due to a multitude of different factors and is dependent on the sperm developmental stage at which damage occurs. Depending on the degree of the damage, lesions may be repaired by the oocyte or the embryo. If this is not possible, permanent damage can ensue, resulting in mutations of the male genome [41] and subsequent diseases including childhood cancer [39,58,60–62].

3. Techniques for evaluating DNA damage in sperm

Male infertility is usually diagnosed by microscopic evaluation of concentration, morphology and motility of sperm in the ejaculate. Most fertility laboratories use sperm isolated by different methods to obtain subpopulations of sperm supposed to have greater fertilisation potential. These tests are essential to provide the fundamental information on which clinicians base their initial diagnosis regarding male fertility [55,63–68]. Nevertheless, in the clinical setting, tests with superior prognostic value are needed. Tests showing much promise are those determining sperm DNA integrity, including the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, sperm chromatin structure assay (SCSA; detecting damage in sperm samples with a high level of DNA fragmentation) [69], and the 8−OHdG immuno-detection cytometry assay (based on the detection of an early marker of DNA oxidation, 8−OHdG) [70], as well as the comet assay. There is also the sperm chromatin dispersion (SCD) test (also known as Halo Sperm assay) that is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops observed in sperm with non-fragmented DNA, following acid denaturation and removal of nuclear proteins [71], and the DNA-breakage detection fluorescence *in situ* hybridisation (DBDFISH) assay that, similarly to the comet assay, analyse agarose-embedded cells but without applying electrophoresis [44,55,69–75].

Although there are some technical differences among the assays mentioned above, they all basically detect sperm with fragmented or damaged DNA. Nevertheless, the comet assay quantifies the level of DNA damage in individual spermatozoa and therefore can be used to determine the degree of heterogeneity of DNA quality in a population of mature sperm [76]. Moreover, the comet assay shows a good correlation with the TUNEL, SCSA and SCD tests [77]. While the specific effects of DNA damage on reproduction remain unclear, sperm nuclear DNA breakage has been positively correlated with several conditions, including lower fertilisation rates in *in vitro* fertilisation (IVF), reduced implantation rates, an increased incidence of spontaneous abortion and disease in offspring, including childhood cancer [61,78,79].

The comet assay is among the methods able to detect germ cell genotoxicity and may be used to demonstrate the ability of a substance or its metabolites to directly interact with the genetic material of sperm. 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 germ cells of humans that can be transmitted to the offspring [80–83]. Although the comet assay also measures some types of DNA damage that could potentially be repaired, there is a good correlation between DNA breaks measured by the comet assay and *in vivo* mutations [84]. It has to be pointed out that currently there are no clinical thresholds for the comet assay, *i.e.* the level of damage leading to infertility has not been established. Nevertheless, since the comet assay is more sensitive compared to other DNA damage detection methods and allows the measurement of DNA damage in individual cells, it is a perfect candidate for the evaluation of a heterogeneous population such as sperm. The comet assay requires a much smaller number of cells compared to other methods making it especially suitable for evaluation of testicular and ejaculated sperm samples from sub-fertile individuals [85,86]. The comet assay with sperm has been extensively used in studies on the effects of environmental substances on fertility, and in connection with male infertility diagnosis and medically assisted human reproduction (Tables 1–5). It is still a matter of debate to what extent the level of sperm DNA damage can affect the possibility of successful human reproduction. The proportion of highly damaged sperm DNA, assessed by the comet assay, has been shown to have predictive value for male infertility and a significant impact in reducing live births [76,87]. Nicopoullos et al. (2019) suggested the use of novel comet assay parameters [High damage Comet Score (HCS) and Low damage Comet Score (LCS)] and introduced threshold levels for the proportion of damaged cells. Using these parameters, they were able to increase the discriminatory power in male infertility diagnosis and predictive power for the success of IVF and intracytoplasmic sperm injection (ICSI) procedures. Therefore, the analysis of sperm DNA strand breaks in the comet assays could add important information to standard semen analysis.

4. Sperm DNA damage assessed by the comet assay

Sperm are quite different from somatic cells in the structure of chromatin. In human sperm, about 85 % of the DNA is packed with protamines in a laminar structure while the remaining 15 % contains histone [88–90]. This indicates that tests intended to analyse DNA damage, such as comet assay, require complete chromatin deprotamination to achieve high sensitivity in the detection of DNA breaks which is also species dependent [91,92]. The protamines contain disulphide bonds, and dithiothreitol (DTT) is usually used in performing the comet assay to break such bonds. However, sperm chromatin is extremely difficult to deproteinize, and high levels of DNA breaks in controls may reflect incomplete unpacking of the DNA. Background levels of DNA vary significantly depending on methodology (particularly depending on lysis conditions). Some protocols employ proteinase K in the lysis step to remove protamines that otherwise impede DNA migration through the agarose. These peculiarities probably limit the sensitivity of the assay in biomonitoring studies (Box 1). Another concern is the choice of positive controls to be used when conducting the sperm comet assay. According to so far published data, positive controls are usually semen samples treated with hydrogen peroxide and X-rays, although researchers have also used glycidamide, benzene and diethylstilboestrol (DES), as well as DNase. A major problem with the sperm comet assay is the lack of a good positive control since the levels of DNA damage obtained with standard SSB inducing agents (hydrogen peroxide and X-rays) differ compared to somatic cells possibly due to the very tight protamine packaging of sperm [92]. Another current problem is the excessive inter-laboratory variability and use of many different protocols; hence, attempts to standardize and harmonize these protocols should be undertaken to enable a direct comparison of results and to establish the sperm comet assay as a valid diagnostic tool [27].

5. Modified comet assay protocols for the evaluation of DNA damage in sperm

There are a few more variants and modifications in addition to the above-mentioned steps.

ReProComet (Repair Proficient Comet) is an *in vitro* method to assess

Table 1
The sperm comet assay for the evaluation of DNA damage in invertebrates.

	Animal	Type of study	Agent/stressor	Concentration range	Parameters tested	Response	References
Molluscs	*Scrobicularia plana*	*In vitro*	B[a]P	B[a]P (10 and 100 μg/L)	% tail DNA	↑	[102]
	Haliotis midae	*In vivo*	H_2O_2	5.5 mmol/L	% tail DNA, OTM, DI	↑	[103]
		In vitro	Cryopreservation, cryoprotectants	freezing-thawing	HL, HA, HI, TotL, TotA, TotI, %TL, %TA, %TI	↑	[106]
	Crassostrea gigas	*In vitro*	Herbicides (diuron, glyphosate and Roundup), H_2O_2 as PC	0.05, 0.1, 0.25, 0.5, and (1.5) μg/L; H_2O_2 (5, 10, 25 and 50 μM)	% tail DNA	↑ (diuron ≥ 0.05 μg/L), Ø (glyphosate), Ø (Roundup), ↑ (H_2O_2 ≥ 25 μM)	[104]
		In vivo	Diuron	Two 7-day exposure pulses at 0.4 and 0.6 μg/L	% tail DNA	↑	[105]
Annelids	*Eisenia fetida*	*In vitro, in vivo*	γ-radiation, X-rays	*In vivo* (^{60}Co γ-radiation (dose rates 0.18–43 mGy/h)), X-rays (41.9 Gy/h), *in vitro* (0.5, 1, 2, 3, 6 or 10 Gy X-rays)	% tail DNA	↑	[98]
	Arenicola marina	*In vivo*	Arsenite	5, 10, 20, 40, and 80 mg As/kg	OTM	↑ (≥ 5 mg As/kg)	[108]
		In vitro	MMS	MMS (18, 32 and 52 mg/L)	% tail DNA	↑ ≥ 32 mg/L	[107]
	Gammarus fossarum	*In vitro, in vivo*	MMS, environmental contaminants ($CdCl_2$, $K_2Cr_2O_7$, paraquat, AMPA and B[a]P)	*In vitro* (1, 2, 10 and 20 mmol/L), *in vivo* (4, 20 and 100 mol/L)	% tail DNA	↑ (*in vitro* ≥ 1 mmol/L), ↑(*in vivo* ≥ 4 mol/L), ↑ ($K_2Cr_2O_7$, paraquat, AMPA)	[109]
		In vivo	MMS	0.8, 2.4, 7 and 22 mg/L	% tail DNA	↑ (≥ 2.4 mg/L)	[112]
		In vivo	Temperature, conductivity		% tail DNA	Ø	[112]
		In vivo, in situ	MMS, $K_2Cr_2O_7$, WWTP	*In vivo* MMS (0.44, 2.2 and 11 mg/L) and $K_2Cr_2O_7$ (0.0625, 0.25 and 1 mg/L), *in situ* (Bourbre River and Bion River (France))	% tail DNA	↑	[110]
		In situ	WWTP	Rhône-Alpes Rivers (France)	% tail DNA	↑	[111]
	Gammarus elvirae	*In vivo*	Hg, Pb	Hg (0.1, 0.5 and 1 μg/L), Pb (25, 50 and 100 μg/L)	% tail DNA	↑ (Hg ≥ 0.1 μg/L), ↑ (Pb ≥ 25 μg/L)	[113]
	Echinogammarus veneris	*In vivo*	Hg, Pb	Hg (0.1, 0.5 and 1 μg/L), Pb (25, 50 and 100 μg/L)	% tail DNA	↑ (Hg ≥ 0.1 μg/L), ↑ (Pb ≥ 25 μg/L)	[113]
	Macrobrachium rosenbergii	*In vivo*	TBT	1, 2 and 4 mg/L	TL, % tail DNA, OTM	↑ (≥ 2 mg/L)	[116]
Arthropods		*In vitro*	Optimisation (H_2O_2, UVC, MMS)	UVC (13.3, 26.5 and 79.5 J/m2), H_2O_2 (5, 25 and 100 μM), MMS (0.5, 1 and 5 mM)	VS, AU	↑	[114]
	Palaemon serratus	*In situ*	Abiotic factors (water temperature), environmental pollution	Seine Bay (Normandy, France)	VS, AU	↑	[115]
		In vivo	Impact of paternal damaged DNA on the reproductive success, MMS (0, 4, 20 and 100 μM)	MMS (0, 4, 20 and 100 μM)	VS, AU	↑	[117]
	Palaemon longirostris	*In situ*	Passive and active recovery of wild specimens	Seine estuary (Normandy, France)	VS, AU	Ø, ↑, ↓ (season dependent)	[118]
	Drosophila simulans	*In vivo*	Wolbachia-infection (ROS)		VS, % tail DNA	↑	[122]
	Paracentrotus lividus	*In vivo*	ZnO NP	Exposed through the diet to different sizes (100 and 14 nm) ZnONPs (1 and 10 mg Zn/kg ZnONPs 100 nm and 1 and 10 mg Zn/kg ZnONPs 14 nm)	% DN	↑	[119]
		In vitro	ZnO NP of different sizes (ZnO Bulk N 200 nm, ZnO NPs 100 nm and ZnO NPs 14 nm) and $ZnSO_4$	0.1, 0.5, 1, 3, 10 and 30 μM [Zn]	VS, SMI	↑	[121]
	Sphaerechinus granularis	*In vitro*	UVB radiation, H_2O_2	UVB radiation (2.2 and 5 kJ/m^2), H_2O_2 (100, 25, 500 and 1000 μM)	% tail DNA	↑ (UVB ≥ 2.2 kJ/m^2), ↑ (H_2O_2 ≥ 100 μM)	[120]

↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % tail DNA; AU, arbitrary units; DN, damaged nuclei; DI, damage index; HA, head area; HI, head intensity; HL, head length; OTM, Olive tail moment; TA, tail area; TI, tail intensity; TL, tail length; TotA, total area; TotI, total intensity; TotL, total length, SMI, sperm mutagenic index; VS, visual scoring; AMPA, aminomethyl phosphonic acid; B[a]P, benzo(a)pyrene; $CdCl_2$, cadmium chloride; H_2O_2, hydrogen peroxide; $K_2Cr_2O_7$, potassium dichromate; MMS, methyl methanesulfonate; NP, nanoparticles; PC, positive control; ROS, reactive oxygen species; TBT, tributyltin chloride; UV, ultra violet; WWTP, wastewater treatment plant effluent; ZnO, zinc oxide; $ZnSO_4$, zinc sulphate.

DNA damage in mammalian sperm. The modification introduced is based on the addition of a crude protein extract from somatic cells, namely from HeLa cells, to agarose-embedded sperm on microscopic slides [93]. The general idea of this approach is that DNA repair enzymes in the protein extract will recognize damage in sperm cells and convert that damage into detectable strand breaks. In order to test the protocol, bull sperm were treated *in vitro* with methyl methanesulfonate (MMS) or melphalan (MLP) and the comet assay was conducted both with and

Table 2
The sperm comet assay for the evaluation of DNA damage in vertebrates.

	Animal	Type of study	Agent/stressor	Concentration range	Parameters tested	Response	References
Cyclostomes	*Petromyzon marinus*	*In vitro, in vivo*	H_2O_2, UV, bisazir, storage conditions	H_2O_2 (1, 10 and 100 μM), UV (860 and 1720 J/m^2), storage conditions (2 and 4 days), bisazir (2 mg/mL)	% head DNA, VS, CS, OTM	↑ ($H_2O_2 \geq 1$ μM), ↑ (UV ≥ 860 J/m^2), ↑(storage conditions ≥ 2 days), ↑ bisazir	[123]
		In vitro	Cryopreservation	Freezing-thawing	% tail DNA, OTM	↑	[124]
		In vitro	Cryopreservation	Freezing-thawing	TM, % of damaged nuclei	↑	[125]
		In vitro	UV, H_2O_2	UV irradiation (2075 mW/cm^2), H_2O_2 (1, 5 and 20 mM), 100 μM H_2O_2 as PC	% tail DNA, OTM	↑ (UV), ↑ (H_2O_2)	[126]
	Oncorhynchus mykiss	*In vitro*	Hg^{2+}, Cd^{2+}	Hg^{2+} (1, 10 and 100 mg/L), Cd^{2+} (10, 100 and 500 mg/L)	% head DNA	↑ ($Hg^{2+} \geq 100$ mg/L), ↑ ($Cd^{2+} \geq$ 10 mg/L)	[128]
		In vitro	Cryopreservation, H_2O_2 as PC	Freezing-thawing, H_2O_2 (500 mM)	% tail DNA	↑	[100]
		In vitro	Cryopreservation (using egg yolk and LDL as additives), H_2O_2 as PC	Freezing-thawing, H_2O_2 (500 mM)	% tail DNA	↑	[129]
		In vitro	Cryopreservation (using egg yolk and LDL as additives), H_2O_2 as PC	Freezing-thawing, H_2O_2 (500 mM)	% tail DNA	↑	[131]
		In vitro	Cryopreservation, H_2O_2 as PC	Freezing-thawing	% tail DNA	↑, Ø (dependent on the location)	[127]
	Salmo trutta	*In vivo*	MMS	MMS (50 mg/kg body weight) previously dissolved in warmed-up coconut oil (2 mL oil/kg body weight)	% tail DNA	↑	[130]
Fishes	*Salvelinus alpinus*	*In vivo*	MMS	MMS (50 mg/kg body weight) previously dissolved in warmed-up coconut oil (2 mL oil/kg body weight)	% tail DNA	↑	[130]
	Acipenser gueldenstaedtii	*In vitro*	Short-term (liquid) storage	Aerobic conditions at 4 °C	% tail DNA, OTM	↑ (dependent on the time of storage)	[132]
	Acipenser baerii	*In vitro*	Short-term (liquid) storage	Aerobic conditions at 4 °C	% tail DNA, OTM	↑ (dependent on the time of storage)	[132]
	Acipenser ruthenus	*In vitro*	Duroquinone	25, 50, 100 and 150 μM	% tail DNA, OTM	↑ (≥ 50 μM)	[133]
	Sparus aurata	*In vitro*	Cryopreservation	Freezing-thawing	% tail DNA, OTM	↑	[124]
	Dicentrarchus labrax	*In vitro*	Cryopreservation, Cryoprotectants	Freezing-thawing (+ taurine and hypotaurine)	% tail DNA	↑ (cryopreservation), ↓ (cryoprotectants)	[135]
		In vitro	Cryopreservation	Freezing-thawing	% tail DNA, TM	↑	[134]
	Pleuronectes vetulus	*In situ*	Wastewater outfall	Orange County (CA, USA)	TM	↑	[136]
	Pleuronichthys verticalis	*In situ*	Wastewater outfall	Orange County (CA, USA)	TM	↑	[136]
	Pseudosciaena crocea	*In vitro*	Cryopreservation, cryoprotectant	Freezing-thawing, DMSO (5, 10, 15, 20, 25 and 30 %)	CL, TL, CR, Dcoe	↑ ≥ 25 %	[137]
	Oreochromis niloticus	*In vivo*	Boron	1, 5, 25, 50 and 100 mg/L	VS	↑ at 100 mg/L	[139]
	Oreochromis niloticus	*In vivo*	$CuSO_4$	1, 2 and 4 mg/kg	% tail DNA	↑	[138]
	Oreochromis niloticus	*In vitro*	Cryopreservation, cryoprotectant (glycerol, methanol and DMSO)	Freezing-thawing	VS	↑ (DMSO) dependent on the cryoprotectant	[140]
	Rana temporaria	*In vitro*	Cryopreservation	Storage conditions	VS	↑ (dependent on the days of storage)	[141]
Amphibians	*Xenopus laevis*	*In vitro*	Validation of the sperm chromatin dispersion test in relation to comet assay		VS	↑	[143]
Reptiles	*Crocodylus porosus*	*In vitro*	Cryopreservation	Freezing-thawing	VS	↑	[142]
	Gallus gallus domesticus	*In vitro*	Cryopreservation, cryoprotectants	Fresh and frozen spermatozoa, cryoprotectants (glycerol 11 %, glycerol 11 % and trehalose (trh) 70 mmol/L, DMA 6%, DMA 6% and trh 70 mmol/L)	% tail DNA, TL, OTM, CL	↑ (DMA medium)	[145]
Birds	*Meleagris gallopavo*	*In vitro*	Liquid storage, $KMnO_4$	$KMnO_4$ (1, 10, 25 and 50 mM)	% head DNA, TL, OTM, CE	↑ (liquid storage), ↑ ($KMnO_4 \geq$ 10 mM)	[146]
	Mericanel della Brianza	*In vitro*	Cryopreservation	Freezing-thawing (−196 °C, 60 °C, 6% DMA at 4 °C)	VS, % tail DNA, TL, CL, OTM	↑	[144]

(*continued on next page*)

Table 2 (*continued*)

	Animal	Type of study	Agent/stressor	Concentration range	Parameters tested	Response	References
	Alectoris barbara	*In vitro*	Cryopreservation, cryoprotectants		% tail DNA, TL, OTM, CL	Ø (among different media)	[145]
	Gyps fulvus	*In vitro*	Cryopreservation	Freezing-thawing	% tail DNA, TL, OTM, CL	Ø	[148]
	Felis silvestris catus	*In vitro*	X-ray system, high-intensity X-ray bursts as PC	16 mrem of radiation exposure	TL	↑ (dependent on the exposure)	[149]
	Prionailurus viverrinus	*In vitro*	X-ray system, high-intensity X-ray bursts as PC	16 mrem of radiation exposure	TL	↑ (dependent on the exposure)	[149]
	Canis lupus familiaris	*In vivo*	Correlation between comet assay *vs* routine assays for the evaluation of semen quality	dogs with abnormal spermiogram *vs* normospermic dogs		↑ (in dogs with non-normospermic semen)	[150]
	Equus caballus	*In vitro*	ROS, cryopreservation	Xanthine (0.3, 0.6 and 1 mM) - xanthine oxidase (0.025, 0.05 and 0.1), freezing-thawing	VS, CS	↑ ROS, ↑ cryopreservation	[156]
		In vitro	Cryopreservation (addition of enzyme scavengers and antioxidants)	Freezing-thawing, CAT (200 U/mL), SOD (200 U/mL), GSH (10 mM), ascorbic acid (10 mM), α-tocopherol (25, 50, 100 and 500 μM and 1 mM)	VS, CS	↑	[155]
		In vitro	Localization of alkali-labile sites		sDFI	↑	[151]
		In vitro	Cryopreservation	Freezing-thawing	VS	↑	[157]
		In vivo	Unilateral orchiectomy	Mild testis stress	HL, % head DNA, % tail DNA, TL, Tmig, TM	↑	[152]
		In vitro	Cryopreservation	Flash-freezing temperature	HL, % tail DNA, TL, OTM, TM, CW	↑	[153]
		In vivo	Redox status	Antioxidant profile	OTM	↑ (low seminal redox status)	[154]
	Equus asinus	*In vitro*	Cryopreservation	Freezing-thawing	sDFI	↑ (dependent on the temperature)	[158]
		In vitro	Localization of alkali-labile sites		sDFI	↑	[151]
Mammals		*In vitro*	Sex-sortement of semen	Dilution, centrifugation, incubation, exposure to DNA stains, laser light	TL, TM, % tail DNA, % head DNA	↑ (conventional samples)	[162]
		In vitro	Cryopreservation, LA	Freezing-thawing, LA (0.125, 0.25, 0.5 and 1 mL)	TL, % tail DNA, TM	Ø	[160]
	Bos taurus	*In vitro*	Cryopreservation, fetuin, hyaluronan, + mixture	Freezing-thawing, hyaluronan (500 μg/mL), fetuin (2.5 mg/mL)	VS, % tail DNA	↑ (cryopreservation), ↓ (fetuin, hyaluronan, + mixture)	[161]
		In vitro	Cryopreservation	Freezing-thawing	% head DNA, TL, OTM	↑	[163]
		In vitro	Cryoprotectants	Glycerol, ethylene glycol, DMSO, + mixture	TL, % tail DNA, TM	↑, ↓ (dependent on the cryoprotectant; no advantages were found in using ethylene glycol or DMSO to replace glycerol)	[159]
	Capra aegagrus	*In vitro*	Cryopreservation, soybean lecithin	Freezing-thawing, lecithin (1, 2, 3, 4, 5 and 6 % wt/vol)	% head DNA, % tail DNA	↑, ↓ (dependent on the lecitin concentration)	[165]
	Capra hircus ancryrensis	*In vitro*	Cryopreservation, raffinose, methionine	Freezing-thawing, raffinose (2.5, 5 and 10 mM), methionine (2.5, 5 and 10 mM)	VS, AU	↑, ↓ (dependent on the concentration)	[166]
		In vitro	Cryopreservation, raffinose, hypotaurine, + mixture	Freezing-thawing, raffinose (10 mM), hypotaurine (5 mM), R+H mixture (5 + 2.5 mM)	VS	↑, ↓	[167]
	Ovis aries	*In vitro*	Cryopreservation	Influence of sperm concentration	VS, AU	↑, ↓ (dependent on the concentration)	[168]
		In vitro	Cryoprotectants (glycerol (G) and ethylene glycol (EG)), trehalose (T)	5% G, 3% G + 60 mM T, 1.5 % G + 100 mM T, 5% EG, 3% EG + 60 mM T, and 1.5% EG + 100 mM T	VS	↑, ↓ (low concentrations of cryoprotectants in combination with T)	[169]
	Sus scrofa	*In vitro*	Cryopreservation, LDL, trehalose, yolk	Freezing-thawing, effects of different extenders (LDL (9%), trehalose (100 mM), yolk (20 %) (v/v))	VS, AU	↑, ↓ (dependent on the extender)	[171]
		In vitro	Cryopreservation, LDL, glycerol, trehalose	Freezing-thawing, LDL, glycerol, trehalose	% tail DNA, CR	↑, ↓ (dependent on the combination)	[170]
				Freezing-thawing	VS	↑	[172]

(*continued on next page*)

Table 2 (*continued*)

Animal	Type of study	Agent/stressor	Concentration range	Parameters tested	Response	References
	In vitro	Cryopreservation (long-term liquid nitrogen storage)				
Tachyglossus aculeatus	*In vitro*	Cryopreservation	Freezing-thawing	VS	↑	[175]
Vombatus ursinus	*In vitro*	H_2O_2	0.003, 0.03 and 0.3 % v/v	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[173]
Macropus giganteus	*In vitro*	H_2O_2	0.003, 0.03 and 0.3 % v/v	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[173]
Phascolarctos cinereus	*In vitro*	H_2O_2	0.003, 0.03 and 0.3 % v/v	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[173]
	In vitro	Cryopreservation	Freezing-thawing	VS, TL	↑	[174]
Diceros bicornis	*In vitro*	Cryopreservation	Freezing-thawing	VS	↑	[176]
Rhinoceros unicornis	*In vitro*	Cryopreservation	Freezing-thawing	VS	↑	[176]
Ceratotherium simum	*In vitro*	Cryopreservation	Freezing-thawing	VS	↑	[176]
Macaca mulatta	*In vitro*	Cryopreservation	Freezing-thawing	% tail DNA	↑	[177]
Alopex lagopus	*In vitro*	Cryopreservation	at +4 °C	VS, % head DNA	↑ (dependent on the time after ejaculation)	[178]
Vulpes vulpes	*In vitro*	Cryopreservation	at +4 °C	VS, % head DNA	↑ (dependent on the time after ejaculation)	[178]
Tursiops truncatus	*In vitro*	Validation	Different incubation times	VS	↑↓ (dependent on the sperm morphotype)	[179]

↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % head DNA; % tail DNA; AU, arbitrary units; CE, comet extent; CL, comet length; CR, comet rate; CS, comet score; CW, comet width; Dcoe, damage coefficient; HL, head length; OTM, Olive tail moment; sDFI, sperm DNA fragmentation index; TL, tail length; TM, tail moment; Tmig, tail migration; VS, visual scoring; CAT, catalase; $CuSO_4$, copper sulphate; DMA, dimethylacetamide; DMSO, dimethyl sulfoxide; GSH, glutathione; H_2O_2, hydrogen peroxide; $KMnO_4$, potassium permanganate; LA, linoleic acid; LDL, low-density lipoprotein; MMS, methylmethanesulfonate; PC, positive control; ROS, reactive oxygen species; SOD, superoxide dismutase; UV, ultra violet.

without addition of a protein extract. Using the standard protocol without protein supplementation, no effect of MMS or MLP was detected on sperm DNA damage. In contrast, a dose-dependent effect was observed after addition of the protein extract, presumably resulting from incision of DNA by DNA repair enzymes at sites of MMS or MPL alkylation. This demonstrates the potential of the protocol to detect damage that is usually not detected by the comet assay.

Many methodologies to evaluate DNA breakage in spermatozoa are unable to differentiate between single-stranded DNA breaks and double-stranded DNA breaks in the same sperm. Hence, a two-tailed comet assay (2T-Comet, employing two dimensional electrophoresis) for assessing DNA damage in spermatozoa was developed to overcome this particular limitation; it is apparently able to simultaneously evaluate DNA single- and double-stranded breaks in human sperm – an approach potentially applicable in the domain of human fertility and andrological pathology [94]. More recently, Cortes-Gutierrez et al. [95] also developed a two-dimensional two-tailed comet assay with a possibility to differentiate between single-stranded and double-stranded DNA breaks in sperm and potential application in fertility assessment.

Besides the above-mentioned modifications, several papers also describe the use of Fpg, EndoIII and/or hOGG1treatment to detect oxidised DNA in sperm of both animals and humans [85,92,96–100] but these assays still need further optimization.

Although the comet assay is usually done on fresh samples, this is impracticable in the case of large epidemiological human studies and so the applicability of using the comet assay on frozen sperm has been assessed; results with cryopreserved and fresh samples compared well, for both animal and human sperm [8,101].

6. The sperm comet assay in animal environmental studies and reproductive toxicology

The sperm comet assay found its role in environmental monitoring from invertebrate to vertebrate species [7,8] with the following studies presented in Tables 1–3.

6.1. Studies performed on invertebrates

Concerning invertebrates, mollusc spermatozoa have been readily used for biomonitoring of both freshwater and marine aquatic environments since those cells are regarded as excellent biomarkers of pollution. Studies were performed both *in vitro* and *in vivo* on different species of bivalves such as peppery furrow shell (*Scrobicularia plana*) [102], abalone (*Haliotis midae*) [103] and oysters (*Crassostrea gigas*) [104–106] with the various aims of optimising the sperm comet assay protocol in bivalves, testing the effects of toxicants, and ensuring adequate fertilisation success to maximize farm production.

Studies have also been carried out in annelid spermatozoa, namely polychaete species of high ecological relevance, such as *Arenicola marina* [107] as well as oligochaetes such as earthworms (*Eisenia fetida*) [98, 108] to test a range of chemical and physical agents both *in vitro* and *in vivo*.

Table 3
The sperm comet assay for the evaluation of DNA damage in laboratory rodents.

Animal	Type of study	Agent/stressor	Concentration range	Parameters tested	Response	References
ICR-CD1 mice	*In vivo*	Lead exposure	0.1, 0.25, 0.5 and 1% lead acetate	% tail DNA, TL, tail motion, OTM	↑ ≥ 0.1 %	[180]
	In vivo	Methamidophos	technical (METt) and commercial (METc) (3.75, 5, and 7 mg/kg bw/day/4 days)	% tail DNA, TM	↑ ≥ 3.75 mg/kg bw/day/4 days	[185]
	In vivo	Methamidophos	3.75 and 5 mg/kg-bw/ip/day/4 days	% tail DNA, TM	↑	[184]
	In vivo	MWCNT, nonylphenol (NP), H_2O_2 as PC	MWCNT (100 mg/kg bw), NP (5 mg/kg bw) + combined, H_2O_2 (100 μmol/L)	% tail DNA, OTM	Ø (NP),↑ (MWCNT), ↑ (combined)	[189]
	In vivo	Chronic acrylamide exposure (amelioration by resveratrol)	Acrylamide (0.18 mg/kg bodyweight/day), resveratrol (10 mg/kg bw/week)	% tail DNA	↑, ↓ (combined)	[181]
	In vivo	Chronic acrylamide exposure	0.001, 0.01, 0.1, 1 and 10 μg/mL (equivalent to 0.0001–2 mg/kg bw/day)	% tail DNA, TM	↑ ≥ 0.001 μg/mL	[182]
Swiss mice	*In vivo*	Methotrexate	5, 10, 20 and 40 mg/kg	TL, % tail DNA, TM, OTM	↑	[187]
	In vivo	Artesunate	13.3 and 40 mg/kg bw	% tail DNA, OTM, VS, DI	↑	[188]
	In vivo	Radiofrequency radiation, melatonin + combined	900 MHz, MEL (5 mg/kg bw/day)	% head DNA, % tail DNA, DI, DF	↑ (RFR), Ø (combined)	[192]
Pzh:Sfis mice	*In vivo*	BPA, X-rays	BPA (5, 10, and 20 mg/kg), X-rays (0.05 Gy), combination (0.05 Gy + 5 mg/kg bw BPA)	% tail DNA	↑, ↑	[183]
F1 mice	*In vivo*	CP, aging	150 mg/kg	% tail DNA, TM	Ø	[186]
A/J mice	*In vivo*	Cigarette smoke	Declared content of 9.4 mg tar and 0.73 mg nicotine	% tail DNA	↑	[190]
C57BL/6 J mice	*In vivo*	X-rays	4 Gy	% tail DNA	↑	[191]
Wistar rats	*In vivo*	Cigarette smoke, amelioration by $ZnCl_2$	Cigarette smoke (20 cigarettes per day), $ZnCl_2$ (20 mg/kg/day) + combined	% tail DNA, TM	↑ (cigarette smoke), Ø ($ZnCl_2$), ↓ (combined)	[196]
	In vivo	Nicotine (effect on offspring)	Pregnant and lactating rats were nicotine-exposed (2 mg/kg/day)	% tail DNA TL, TM, OTM	↑	[195]
	In vivo	Sertraline	5, 10 and 20 mg/kg/day	TM	↑	[198]
	In vivo	Citalopram hydrobromide	5, 10 and 20 mg/kg/day	TM	↑	[197]
	In vivo	Levetiracetam	50, 150 and 300 mg/kg/day	TM	↑ ≥ 150 mg/kg/day	[199]
	In vivo	Nandrolone decanoate, amelioration by taurine	ND (10 mg/kg/week), T (100 mg/kg/day) + combined	% tail DNA, TL, TM, OTM	↑ (ND), Ø (T), ↓ (combined)	[201]
	In vivo	Fipronil	2.5, 5 and 10 mg/kg/day	% tail DNA, TL, TM, OTM, AU	↑ ≥ 2.5 mg/kg/day	[202]
	In vivo	AgNPs	5 or 10 mg/kg of 20 nm or 200 nm AgNPs	% tail DNA, % head DNA, TL	↑, Ø (size-, dose- and time-dependent)	[206]
	In vivo	Depleted Uranium (F0 and F1 generation)	4 and 40 mg/kg/day	% tail DNA, TL, TM, OTM	↑	[207]
	In vivo	Electromagnetic field (EMF)	3 G mobile phone radiation (1900−2170 MHz)	% tail DNA, TL, TM, OTM (+ large number of other parameters)	↑	[208]
	In vivo	Doxorubicin	1.25, 2.5 and 5 mg/kg weekly	% tail DNA, TL, TM, tailed cells	↑ ≥ 1.25 mg/kg/weekly	[200]
	In vivo	Arsenic, amelioration by quercetin	As (50 ppm), QU (50 mg/kg bw)	CL, % head DNA, TL, % tail DNA, TM, OTM	↑ (As), Ø (combined)	[193]
	In vivo	$HgCl_2$, amelioration by *Chenopodium album Linn.* and Vit C	$HgCl_2$ (0.15 mg/kg bw), Vit C (200 mg/kg bw), C. album (200 mg/kg bw)	HL, % head DNA, TL, % tail DNA, TM	↑ ($HgCl_2$), Ø (*C. album*), ↑Ø (combined) dependent on the descriptor	[194]
Sprague Dawley rats	*In vivo*	BPA, amelioration by MEL	BPA (200 mg/kg bw /day), MEL (10 mg/kg bw/day)	% tail DNA, TL	↑ (BPA), Ø (MEL, combined)	[204]
	In vivo	Aroclor 1254, impact of Se	Aroclor (10 mg/kg), Se (<0.05 mg/kg and 1 mg/kg diet)	% tail DNA	↑ (Aroclor, SeD, A + SeD), Ø (SeS, A + SeS)	[205]
	In vivo	High-fat diet, protective effect of probiotics	Normal standard diet (5% fat, w/w), high-fat diet (20 % fat, w/w), high-fat diet + 2% probiotics (w/w)	% tail DNA, TL, TEM, OTM	↑ (high-fat diet), ↓ (probiotics)	[210]
	In vivo	STZ-induced diabetic rats, amelioration by telmisartan (T)	STZ (55 mg/kg), T (3, 6 and 12 mg/kg/day)	% tail DNA, TL, TM, OTM	↑ (STZ), Ø (T), ↓ (STZ + T)	[209]
Holtzman rats	*In vivo*	BPA	10 μg and 5 mg/kg/bw	% tail DNA, TL, TM, OTM	↑ ≥ 5 mg/kg/bw	[203]
Zucker rat	*In vivo*	Obesity	Lean *vs* fat	% tail DNA, TL, TM, OTM	↑ (fat)	[211]
Mesocricetus auratus	*In vivo*	Protection of sperm DNA by accessory sex gland secretions	NADPH-induced OS (1.25, 2.5, 5, 10 and 20 mmol/L)	TM	Male accessory sex gland secretions can preserve the integrity of the sperm genome	[213]
	In vivo	Protective role of SOD, CAT, GPx or GSH-Px	NADPH-induced OS (1.25, 2.5, 5, 10 and 20 mmol/L)	Number of comets per 10 000 sperm	↑, ↓ (dependent on the enzyme)	[212]
Oryctolagus cuniculus	*In vivo, in vitro*	RISUG® (Reversible Inhibition of Sperm Under Guidance), H_2O_2 as PC	Vas occlusion with RISUG® for 3 and 12 months, reversal with DMSO and $NaHCO_3$ after 3 and 12 months, H_2O_2 (20 μM)	% tail DNA, CL, OTM	Ø, ↑ (H_2O_2)	[214]

↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % head DNA; % tail DNA; AU, arbitrary units; CL, comet length; DF, damage frequency; DI, damage index; HL, head length; OTM, Olive tail moment; TEM, tail extent moment; TL, tail length; TM, tail moment; VS, visual scoring; BPA, bisphenol A; CAT, catalase; CP, cyclophosphamide; DMSO, dimethyl sulfoxide; EMF, electromagnetic field; GPx, glutathione peroxidase; GSH, glutathione; H_2O_2, hydrogen peroxide; $HgCl_2$, mercury chloride; MEL, melatonin; MWCNT, multi-walled carbon nanotubes; $NaHCO_3$, sodium bicarbonate; NP, nanoparticles; OS, oxidative stress; PC, positive control; SOD, superoxide dismutase; STZ, streptozotocin.

Table 4
The sperm comet assay for the evaluation of DNA damage in human biomonitoring.

Agent/stressor	Exposure/setting	Parameters tested	Response	References
Urinary metal in infertile men	As, Cd, Co, Cr, Cu, Fe, Pb, Mn, Mo, Hg, Ni, Se, Zn	% tail DNA, TL, TM	Hg ↑, Ni ↑, Mn ↑	[216]
Environmental and occupational boron exposure	<100 ng B/g blood, ≥ 651 ng B/g blood	% tail DNA	Ø	[218]
Boron-exposed workers, H_2O_2 as PC	<100 ng B/g blood, > 150 ng B/g blood, H_2O_2 (300 μM)	% tail DNA	Ø	[217]
Occupational benzene exposure	0–5, 5–10 and 10–15 y of exposure for 8 h/day	TL	↑	[219]
Occupational styrene exposure	At least 2 y in the last 5 y and continuously for 6 months in factories producing reinforced plastics	% tail DNA, OTM	↑	[221]
Occupational styrene exposure	At least 2 y in the last 5 y and continuously for 6 months in factories producing reinforced plastics	% tail DNA	↑	[220]
Occupational acrylonitrile (ACN) exposure	Exposure-time was 2.8 y. The mean concentration of ACN was 0.8 ± 0.25 mg/m^3 at operation sites.	TL, rates of comet sperm	↑	[222]
Environmental exposures to phthalates	8 urinary phthalate metabolites—MEP, monomethyl phthalate (MMP), MEHP, MBP, MBzP, mono-n-octyl phthalate (MOP), mono-3-methyl-5-dimethylhexyl (isononyl) phthalate (MINP), and monocyclohexyl phthalate (MCHP)	% tail DNA, TL, TDM	↑	[225]
Semen phthalate metabolites	8 phthalate metabolites [monomethyl phthalate (MMP), monoethyl phthalate (MEP), MBP, mono-n-octyl phthalate (MOP), monobenzyl phthalate (MBzP), MEHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)]	% tail DNA, TDM	↑	[224]
Exposure to PBDEs or phthalates	Hair PBDE and urinary phthalate	% tail DNA, OTM	Ø	[223]
Occupational fenvalerate exposure	Production area workers	% tail DNA, TDM, CE	↑	[226]
Urinary levels of insecticide metabolites	Environmental exposures to carbaryl and chlorpyrifos	% tail DNA	↑	[227]
Environmental exposure to PAHs	Urinary polycyclic aromatic hydrocarbon metabolites (2-OHNa, 9-OHPh, 2-OHFlu, and 1-OHP)	% tail DNA, TL, TDM	↑	[228]
Occupational exposure to ionising radiation	Diagnostic or therapeutic radiation (X-, β- and γ-rays) facilities	% tail DNA, % head DNA, OTM, VS	↑	[229]
Shock-wave lithotripsy for distal ureteral stones	Men who had undergone SWL for distal and upper ureter stones	VS, AU	↑	[230]
Cigarette smoking	Smoking cigarettes on a regular basis	% DNA fragmentation	Ø	[232]
Cigarette smoking	At least 10 cigarettes/day	% tail DNA, TL	↑	[231]
Sleep duration	Longer (> 9.0 h/day) or shorter (≤ 6.5 h/day)	% tail DNA	Ø	[233]
Lifestyle factors	Aging (22–80-y), micronutrients intake (Vit C, Vit E, β-carotene, zinc, and folate)	% tail DNA, VS	↑, Ø, ↓	[234]
Aging, caffeine consumption	Mean age: 46.4 y, range: 22–80 y, >3 cups coffee/day	% tail DNA, OTM, TEM	↑ (aging), ↑ (caffeine consumption)	[235]
Cancer patients	Testicular cancer and lymphoma	% tail DNA	↑	[239]
Different clinical groups	Aged 18–50 y with 26 fertile and 154 infertile	Fragmented and non-fragmented sperm	↑ (infertile)	[240]
Kartagener's syndrome	Kartagener's syndrome with four failures of fertilisation	CT, VS	↑	[244]
Varicocelectomy	Adolescents (14–19 y), Tanner stages IV or V with varicocele grades II or III	VS	↓	[245]
Effect of varicocele	Clinically diagnosed varicocele of grade II or III	VS	↑	[243]
Different clinical groups, H_2O_2 as PC	Asthenoteratozoospermic (ATZ) with or without varicocele, oligoasthenoteratozoospermic (OATZ) or balanced chromosome rearrangements, H_2O_2 (0.03, 0.15 and 0.30 %)	% SDF	↑ (OATZ, ATZ and ATZ with varicocele)	[241]
Unexplained recurrent miscarriage in couples without a female factor, DNAse as PC	Semen samples from recurrent pregnancy loss (RPL) couples	% SDF	↑ (RPL patients)	[246]
Effect of sperm nuclear vacuoles	Sperm categorized by Motile Sperm Organelle Morphology Examination (MSOME) Grades	VS	↑	[247]

↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % head DNA; % tail DNA; AU, arbitrary units; CE, comet extent; CT, comet tail; OTM, Olive tail moment; SDF, sperm DNA fragmentation; TDM, tail distributed moment, TEM, tail extent moment; TL, tail length; TM, tail moment; VS, visual scoring; ACN, acrylonitrile; ATZ, asthenoteratozoospermic; H_2O_2, hydrogen peroxide; MSOME, Motile Sperm Organelle Morphology Examination; OATZ, oligoasthenoteratozoospermic; PAHs, polycyclic aromatic hydrocarbons; PBDEs, polybrominated diphenyl ethers; PC, positive control; RPL, recurrent pregnancy loss.

Crustacean spermatozoa can also serve as a good model in ecotoxicology and biomonitoring. Spermatozoa of several amphipod crustaceans, such as *Gammarus fossarum* [109–112], *Gammarus elvirae* [113] and *Echinogammarus veneri* [113], as well as prawns and shrimps (*Macrobrachium rosenbergii*, *Palaemon serratus* and *Palaemon longirostris*) [114–118] were used in order to optimize the protocol or to test different toxicants as well as abiotic factors in laboratory conditions or in the field. Results also showed that spermatozoa appeared significantly more susceptible than oocytes to genotoxicants. It was demonstrated that a significant correlation exists between the level of sperm DNA damage of exposed adults and the abnormality rate in progeny embryos. Moreover, reproduction defects were observed at levels of DNA damage exceeding a minimal threshold, which could have significant consequences for the population dynamics of this ecologically relevant species [110]. Several species of sea urchins (*Paracentrotus lividus* and *Sphaerechinus granularis*) and their spermatozoa have also been used for the assessment of DNA damage resulting from both physical and chemical agents in the marine environment [119–121].

Table 5
The sperm comet assay for the evaluation of DNA damage in human sperm *in vitro* and for cryopreservation research.

Agent/stressor	Exposure/setting	Parameters tested	Response	References
B[a]P, BPDE, smokers *vs* non-smokers, X-rays as PC	B[a]P (1, 5, 10 and 25 μM), BPDE (5, 20 and 50 μM), X-rays (200 Gy)	% tail DNA	↑,Ø (B[a]P), ↑,Ø (BPDE), ↑ (smokers), ↑ (X-rays)	[96]
MMS	0.3, 0.6, 0.8 and 1.2 mM	% tail DNA, OTM	↑ ≥ 0.3 mM	[248]
Perfluoroalkyl substances (PFAS), H_2O_2 as PC	Perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorohexanoic acid (PFHxA) (0.1–1 mM), H_2O_2 (600 μM)	% tail DNA	Ø, ↑ (H_2O_2)	[249]
Estrogenic compounds, H_2O_2 as PC	Diethylstilboestrol, progesterone, 17β-oestradiol, noradrenaline and triiodotyronine, butylated hydroxyanisol (BHA) (500 μM), H_2O_2 (50 μM)	OTM	↑ (estrogenic compounds), ↑ (H_2O_2), Ø (BHA)	[99]
Inhalation anesthetics	Halothane, isoflurane, sevoflurane and desflurane (0.1, 1, 10 and 100 mM)	% tail DNA	↑ (halothane), ↑ (isoflurane), ↑ (sevoflurane), Ø (desflurane)	[250]
Doxorubicin	0.2, 0.4, 0.8 and 1.6 μM	% head DNA, TM	↑ (0.8 μM)	[251]
Antimalarials, H_2O_2 as PC	Cryptolepine and its analogues (2,7-dibromocryptolepine, 2-fluorocryptolepine, 7-bromo-8-nitrocryptolepine and 8-chloro-7-nitrocryptolepine) (100, 200 and 300 μM), H_2O_2 (100 μM)	% tail DNA, OTM	Ø (antimalarials), ↑ (H_2O_2)	[252]
Food additives	Citric acid (CA), benzoic acid (BA), brilliant blue (BB), sunset yellow (SY) (50, 100, 200 and 500 μg/mL)	% tail DNA, TL, TM	↑ (CA, 500 μg/mL), ↑ (BA, 500 μg/mL), ↑ (BB, 200 μg/mL), ↑ (SY, 200 μg/mL)	[253]
Cypermethrin, protective role of Vit C and E	Cypermethrin (10 μM), Vit C (20 mM), Vit E (2 mM)	% tail DNA, TL, TM	↑ (cypermethrin), ↓ (Vit)	[254]
Nicotine	0.25, 0.5 and 0.75 mM	Comet values (%)	↑ (0.5 mM)	[255]
Drinking water disinfection by-products, haloacetic acids (HAAs)	Iodoacetic acid (IAA), bromoacetic acid (BAA), chloroacetic acid (CAA), butylated hydroxanisole (BHA) and catalase as protectors	% tail DNA, OTM	↑ (HAAs), ↓ (BHA and catalase)	[256]
Cerium dioxide nanoparticles, H_2O_2 as PC	CeO_2-NP (0.01, 0.1, 1 and 10 mg/L), H_2O_2 (110 μM)	% tail DNA	↑	[258]
Titanium dioxide nanoparticles, benzene as PC	TiO_2-NP (1 and 10 μg/L), benzene (0.4 μL/mL)	% tail DNA	↑	[257]
Radiation	^{137}Cs gamma rays (25, 50, 75 and 100 Gy)	% tail DNA, TM	↑	[259]
Inflammatory mediators	Leukocytes, combinations of pro-inflammatory cytokines (interleukin [IL]-6 + IL-8 and IL-12 + IL-18), bacterial strains (*Escherichia coli* and *Bacteroides ureolyticus*)	CL	↑	[260]
Dietary/environmental compounds, H_2O_2 as PC	Heterocyclic amines, organochlorines, PAHs, mycotoxins, lipid peroxidation products, alcohol, H_2O_2 (10, 50 and 100 μM)	% tail DNA, OTM	↑	[261]
Cryopreservation	Liquid nitrogen (flash freezing with and without cryopreservative, programmable freezing with and without cryopreservative)	CL	↑ (freezing), ↓ (freezing + cryopreservative)	[101]
Cryopreservation	Cryopreservation in patients with teratospermia	OTM	↑ (teratospermic samples)	[265]
Cryopreservation	Normospermic and four categories of infertile males	OTM, % tail DNA, % head DNA, comet number, intact DNA number, CL, TL, TM	↑ (teratospermic and astheno-teratozoosspermic)	[264]
Cryopreservation	Fresh *vs* cryopreserved (fertility status)	% SDF	↑, Ø	[262]
Cryodamage, protective role of acetyl-L-carnitine	Freezing/thawing, acetyl-L-carnitine (2.5, 7.5, 15 mmol/L)	CR, % tail DNA, TL, OTM	↑ (cryodamage), ↓ (acetyl-L-carnitine)	[266]
H_2O_2, protective role of gangliosides	H_2O_2 (200 μM), trisialogangliosides (GT1b) (100 μM)	TL, TM	↑ (H_2O_2), Ø, ↓ (GT1b)	[267]
Cryopreservation, protective role of gangliosides	Freezing/thawing, monosialogangliosides (GM1), trisialogangliosides (GT1b) (100 μM)	% tail DNA, TM	↑ (cryopreservation), Ø, ↓ (GM1, GT1b)	[263]
Cryopreservation, protective role of gangliosides	Freezing/thawing, monosialogangliosides (GM1), trisialogangliosides (GT1b) (100 μM)	TM	↑ (cryopreservation), Ø, ↓ (GM1, GT1b)	[268]
Cryopreservation, protective role of genistein	Freezing/thawing, genistein (1 and 10 μmol/L)	% head DNA, % tail DNA, TL, TM, OTM	↑ (cryopreservation), ↓ (genistein)	[269]
Oxidation Stress, protective role of oleoylethanolamide (OEA)	OEA (2.5 nM)	% tail DNA, TM	↓ (OEA)	[270]
Oxidation Stress, protective role of isoflavone	H_2O_2 (500 μmol/L), genistein and equol (0.01–100 μmol/L), ascorbic acid (10–600 μmol/L), α-tocopherol (1–100 μmol/L)	TM	↑ (H_2O_2), ↓ (antioxidants)	[272]
Thyroid hormone and noradrenaline induced DNA damage, antioxidants, diethylstilboestrol (DES) as PC	Triiodothyronine (T3, 80 μM), L-thyroxine sodium salt (T4, 80 μM), noradrenaline (NA, 300 μM), DES (175 μM), antioxidant (catalase, kaempferol, quercetin)	% head DNA	↑ (steroidal estrogens), ↓ (protectors)	[271]

↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % head DNA; % tail DNA; CL, comet length; CR, comet rate; OTM, Olive tail moment; SDF, sperm DNA fragmentation; TL, tail length; TM, tail moment; BA, benzoic acid; B[a]P, benzo(a)pyrene; BB, brilliant blue; BHA, butylated hydroxyanisol; BPDE, benzo[*a*]pyrene-7,8-diol-910-epoxide; CA, citric acid; CeO_2, cerium dioxide; DES, diethylstilboestrol; GM1, monosialogangliosides; GT1b, trisialogangliosides; H_2O_2, hydrogen peroxide; MMS, methylmethanesulfonate; NP, nanoparticles; OEA, oleoylethanolamide; PAHs, polycyclic aromatic hydrocarbons; PC, positive control; PFAS, perfluoroalkyl substances; SY, sunset yellow; TiO_2, titanium dioxide.

The comet assay was also employed on insect spermatocytes, namely in fruit fly (*Drosophila*), to test molecular interactions between symbiotic bacteria and their animal hosts. Results showed that disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of Wolbachia-infected *Drosophila simulans* [122].

6.2. Studies performed on vertebrates

As for vertebrate species, besides environmental studies conducted in order to assess the impact of different contaminants *in vitro* and *in vivo*, the sperm comet assay has been used with regard to animal reproduction especially in species that have commercial value. The comet assay was applied to sea lamprey (*Petromyzon marinus*) sperm to monitor effects of environmental disturbances and to test the impact of the toxicant bisazir, with the results of this study indicating a genotoxic effect. Thus, this study demonstrated the successful application of the comet assay to monitor environmental effects in sea lamprey spermatozoa and possibly other species of ancient fish with acrosomal sperm [123]. The sperm comet assay has been used to evaluate ecotoxicity in a variety of fresh water and marine fish, and also to test the possible impact of freezing and thawing on DNA integrity, especially in species that have commercial value such as trout (*Oncorhynchus mykiss, Salmo trutta*) [100,124–131], charr (*Salvelinus alpinus*) [130], sturgeon (*Acipenser gueldenstaedtii*, *Acipenser baerii* and *Acipenser ruthenus*) [132,133], sea bream (*Sparus aurata*) [124], sea bass (*Dicentrarchus labrax*) [134,135], flatfish (*Pleuronectes vetulus* and *Pleuronichthys verticalis*) [136], yellow croaker (*Pseudosciaena crocea*) [137] and tilapia (*Oreochromis niloticus*) [138–140].

In amphibians, the effects of storage and cryopreservation on sperm DNA damage were also assessed using the comet assay. In the European common frog (*Rana temporaria*) it was shown that sperm DNA damage increased during refrigerated storage although this did not affect spermatozoal motility or fertility [141]. The effect of cryopreservation on sperm DNA integrity in the crocodile (*Crocodylus porosus*) was examined using a two-tailed comet assay [142]. The comet assay was applied to spermatozoa of the African clawed frog (*Xenopus laevis*) for the validation of other DNA damaging assays [143].

The comet assay was also used for the evaluation of DNA damage in poultry (chicken and turkey) spermatozoa during cryopreservation and short-term liquid storage, which are crucial in terms of artificial insemination as well as storage in cryobanks. [144–147]. Effects of cryopreservation were also evaluated in vultures (*Gyps fulvus*) and no induction of DNA damage was observed during the process, which could be beneficial in the griffon conservation programs [148].

The sperm comet assay has also been used in pets, namely cats and dogs for the detection of DNA damage in their sperm induced by X-rays as well as for assessing semen quality [149,150]. The sperm comet assay was also conducted on several other domestic animals such as horses [151–157], donkeys [151,158], bulls [159–164], goats [165,166], sheep [167–169] and boars [170–172] to test semen quality for cryopreservation and artificial insemination.

The sperm comet assay has been applied to wild animals as well. Sperm DNA integrity was evaluated in several metatherian species, namely in echidna (*Tachyglossus aculeatus*), wombat (*Vombatus ursinus*), koala (*Phascolarctos cinereus*) and kangaroo (*Macropus giganteus*), indicating that the sperm DNA of the marsupial species is more sensitive to oxidative stress insults compared to eutherian species [173–175].

As for large wildlife mammals, the sperm comet assay was employed to assess DNA damage in three rhinoceros species (*Diceros bicornis*, *Rhinoceros unicornis* and *Ceratotherium simum*) and discovered the presence of DNA damage shortly after thawing the sperm [176]. A similar effect was found for monkey (*Macaca mulatta*) sperm following freezing–thawing [177]. Analysis of sperm chromatin structure in foxes (*Alopex lagopus* and *Vulpes vulpes*) showed that the genetic material of the sperm of silver foxes was more stable compared to the arctic ones, suggesting that analysis of chromatin stability should complement morphological and molecular evaluation of semen [178]. In addition, similar tests have been carried out with marine mammals; the comet assay was used for the evaluation of sperm DNA integrity of dolphins (*Tursiops truncatus*) both in the field and in laboratory settings [179].

6.3. Studies performed on laboratory animals

In laboratory rodents such as mice and rats, the sperm comet assay has been used for the evaluation of effects of various chemical and physical agents. In mice, the assay was performed in order to test the effects of heavy metals [180], acrylamide [181,182], bisphenol A [183], pesticides [184,185], anticancer drugs [186,187], antimalarial drugs [188], nanotubes [189], cigarette smoke [190], X-rays [183,191], gamma and radiofrequency radiation [192]. The sperm comet assay was used to test protective effects of different substances and natural products [181,192] as well as the influence of aging [186]. Similar studies were conducted in rat sperm and evaluated the DNA damaging effects of arsenic [193], mercury [194], cigarette smoke and nicotine [195,196], antidepressants [197,198], antiepileptic drugs [199], anticancer drugs [200], anabolic steroids [201], pesticides [202], bisphenol A [203,204], polychlorinated biphenyls [205], nanoparticles [206], depleted uranium [207], electromagnetic irradiation [208]. The sperm comet assay was used to test protective effects of different substances and natural products also in rats [193,194,201,204,209,210], as well as the impact of diet and obesity [210,211]. Besides mice and rats there are also several reports of studies performed on hamsters and rabbits [212–214].

As described above, the comet assay is extensively used in animals, especially with regard to evaluating the impact of cryopreservation on sperm DNA integrity, because the process of cryopreservation is crucial for artificial insemination and for the conservation of genetic resources in cryobanks. Sperm DNA integrity may be impaired during cryopreservation; however, the damages induced on nuclear DNA are minor compared to the damages produced on other cellular characteristics, such as membrane integrity and motility. Although there are results indicating that an increase in DNA damage occurs during cryopreservation, depending on the conditions as well as species used, they also suggest a low sensitivity of vertebrate spermatozoa to DNA breakage that should not be considered as a major cause of sperm injuries during cryopreservation. Nevertheless, given the unusually high incidence of DNA damage in the sperm of some individual animals after cryopreservation and thawing, the standardization of a methodology to assess sperm DNA damage in such animals could contribute to male reproductive management of highly endangered species [8,144,176,215].

7. The sperm comet assay in human biomonitoring and reproductive toxicology

Studies reporting results from sperm comet assay, used either for the purpose of human biomonitoring or for human reproductive toxicology, are presented in Tables 4 and 5.

7.1. Use of the sperm comet assay in human biomonitoring

For the purpose of human biomonitoring, the sperm comet assay has been employed to test the effects of either environmental or occupational exposure to heavy metals [216], boron [217,218], benzene [219], styrene [220,221], acrylonitrile [222], phthalates [223–225], pesticides [226,227], polycyclic aromatic hydrocarbons [228], ionising radiation [229], shock-wave lithotripsy [230], as well as some lifestyle habits such as smoking [231,232], sleep duration [233] or micronutrients intake [234]. Schmid et al. [235] investigated the associations between male age and sperm DNA damage and the influence of several lifestyle factors in healthy males and found that older men have increased sperm DNA damage. In addition, men with substantial daily caffeine consumption had increased sperm DNA damage. The authors concluded that induced DNA damage could be converted to chromosomal aberrations and gene mutations after fertilisation, hence increasing the risks of developmental defects and genetic diseases among offspring. The above studies and other available literature indicate that several environmental and occupational agents, as well as certain lifestyle habits such as smoking, may affect sperm DNA integrity [236–238].

The comet assay has also been used to test sperm DNA integrity following cancer diagnosis, revealing increased DNA damage in cancer patients compared with fertile healthy donors [239]; and a comparative study of fertile and infertile men indicated higher sperm DNA breakage in the infertile subjects [240]. Different clinical groups of patients showed different sperm DNA damage profiles compared with healthy fertile donors [241–246]. In addition, the comet assay was used to assess the degree of DNA damage in sperm categorized by Motile Sperm Organelle Morphology Examination (MSOME) grades, showing that lower MSOME grading correlated with lower sperm DNA damage. Therefore, the observation of sperm nuclear vacuoles using real-time optical microscopy without precise DNA damage examination is not sufficient for optimal sperm selection for ICSI [247].

7.2. Use of the sperm comet assay for in vitro studies and cryopreservation research

In order to investigate the toxicological properties of different chemical and physical agents and the association between such exposures and male reproductive toxicity, sperm have been exposed *in vitro*. Regarding reproductive toxicity, studies have focused on effects of benzo[*a*]pyrene [96], MMS [248], perfluoroalkyl and polyfluoroalkyl substances [249], estrogenic compounds [99], anaesthetics [250], anticancer drugs [251], antimalarial drugs [252], food additives [253], pesticides [254], nicotine [255], drinking water disinfection by-products [256], nanoparticles [257,258], gamma rays [259], inflammatory factors [260] and other dietary/environmental compounds [261] (see Table 5).

Furthermore, several studies have shown that the process of cryopreservation could induce sperm DNA damage, also indicating that oxidative stress is the major effector in DNA damage during sample cryopreservation [262–265]. Thus, there is a certain risk of decreased fertility after using a frozen sample, but no evidence for increased miscarriage risk from cryopreserved spermatozoa [262]. Moreover, Duty et al. [101] found that flash-freezing in liquid nitrogen without addition of cryopreservative gave most similar results to fresh human semen samples, and so this represents the most appropriate cryopreservation method for human semen in epidemiological studies.

Moreover, *in vitro* studies have been carried out in order to evaluate protective effects of different substances against cryopreservation, chemicals and ROS induced DNA damage in sperm. Several compounds showed a good capability to protect the genetic stability of human spermatozoa, such as acetyl-L-carnitine [266], gangliosides [263,267,268], genistein [269], oleoylethanolamide [270], and flavonoids [271,272], as well as vitamins C and E [254].

8. Biological and clinical significance of DNA damage in sperm

DNA damage and its accumulation constitute a common property of sperm, and an increase in the level of sperm DNA breakage is known to influence natural reproduction. It has to be pointed out that sperm DNA damage can arise *via* a multitude of factors and the consequences are dependent on the stage of sperm development at which damage occurs. For instance, damage in sperm due to a single exposure will disappear without any effects if that 'generation' of sperm is not used for making progeny. Damage in spermatogonia (sperm-stem cells) can, however, lead to mutations that can in theory be transmitted to any offspring after the mutation occurred, because sperm derived from such spermatogonia may transmit that mutation to the oocyte. Fortunately, spermatogonia are DNA repair proficient and the initial DNA damage in spermatogonia may no longer be detectable in mature sperm. Although sperm lose the ability to repair their DNA during maturation, some types of lesions in sperm DNA may be repaired in the oocyte. If this is not possible, damage may be converted into a permanent base change, leading to mutations of the male genome in all descendent cells. As a result, when repair in the oocyte or the embryo is not error free, DNA damage derived from the male genome may lead to an early abortion. Furthermore, sperm DNA damage and fragmentation may in general be the cause of numerous diseases, including childhood cancer [39,58,60–62,273].

The evaluation of sperm chromatin and DNA structure was initially undertaken to improve our understanding of spermatogenesis, sperm physiology, sensitivity to reproductive toxicants and reproductive biology [274–278]. The effects of sperm DNA damage on male infertility and assisted reproductive treatment outcomes are still a matter of debate, although there is sufficient evidence in the existing literature to conclude that sperm DNA damage is associated with a negative effect on successful clinical pregnancy following IVF and/or ICSI treatment [279,280]. Therefore, different sperm chromatin and DNA tests, including the comet assay, have been used in the evaluation of infertile men in order to provide a more accurate and in depth diagnosis than offered by standard sperm parameters alone, such as sperm concentration, motility and/or morphology [274,281,282]. Sperm DNA damage assays have also been studied in the context of assisted reproductive technologies (ARTs) to evaluate their capacity to predict pregnancy outcome after assisted reproduction.

Generally, it has been described that different types of sperm DNA breaks (single- and double-stranded DNA breaks) cause different clinical reproductive effects. Single-stranded DNA breaks that are present extensively as multiple break points in all regions of the genome, are related to oxidative stress and cause a lack of clinical pregnancy or an increase of the conception time. Contrary to that, double-stranded DNA breaks that are mainly localized and attached to the nuclear matrix, are possibly related to a lack of DNA repair in meiosis consequently causing a higher risk of miscarriage and low embryo quality, as well as higher risk of implantation failure in ICSI cycles [62]. It has also been suggested that double-stranded DNA breaks have a stronger negative impact on reproductive outcomes such as fertilisation, implantation, pregnancy and miscarriage as well as live birth rates compared to single-stranded breaks. Nevertheless, future research are still needed to fully elucidate effects of sperm single and double-stranded DNA breaks on reproduction and their relevance in clinical setting [32]. Although studies done so far have not shown a clear association between sperm DNA and pregnancy outcomes after ICSI, it cannot be excluded that very high levels of DNA damage will influence ICSI outcomes. In couples undergoing either IVF or ICSI, there is evidence suggesting that sperm DNA damage is associated with an increased risk of pregnancy loss [283,284]. The predictive value of standard sperm parameters is limited, as they do not address the integrity of the genome contained in the sperm head, which is essential for the success of natural or assisted fertilisation, normal embryo development, as well as foetus and child development. Although the clinical usefulness of tests of sperm DNA damage needs to be more thoroughly assessed, the available data indicate that there is clinical value in testing couples prior to assisted reproductive technologies and also couples with repeated miscarriages, although such assays are still not routinely used in a clinical setting [85,274,283,285]. The drawback of the comet assay is that the sperm cannot be used for fertilisation purposes after the test has been performed, but for the purpose of explaining repeated miscarriages or the impossibility of conception it is very promising.

Several studies have evaluated the significance of sperm DNA damage, suggesting an association between the level of DNA damage and adverse effects on semen parameters and reproduction. For instance, a number of studies showed that infertile men have markedly higher levels of sperm chromatin damage and DNA damage compared with fertile men [240,274,286–289]. DNA of semen and prepared spermatozoa from fertile men was found to be unaffected by cryopreservation, whereas spermatozoa from infertile men were significantly damaged by the freeze-thawing procedure suggesting negative effects of cryopreservation predominantly in infertile men [290]. Furthermore, an increased level of sperm DNA damage in the processed ejaculate affected embryo metabolism, which could be related to embryonic genetic integrity [291]. It was also shown that double-stranded breaks in sperm

DNA caused a delay in embryo development and impaired implantation, while single-stranded DNA damage did not significantly affect embryo kinetics and implantation [292].

As for the role of the comet assay, sperm DNA damage assessed by this technique has a close inverse relationship with live-birth rates after IVF [293]. It was suggested that evaluation of sperm DNA by the comet assay may be of value in fertility research [294]. More recently, it was proposed that the novel use of comet parameters of sperm DNA damage might increase the assay's utility for diagnosing male infertility and predicting live births following both IVF and ICSI. This was studied by quantifying low, high and average damage levels in semen samples from fertile donors and comparing them with men attending fertility treatments. The authors concluded that the proportion of sperm with low or high levels of DNA damage provides discriminatory information for male infertility diagnosis and prediction of both IVF and ICSI live births, hence providing a more personalised male fertility diagnosis and treatment in couples with unexplained and male infertility [76].

There is some evidence for transgenerational changes in genetic integrity in preimplantation embryos in response to fertilisation with DNA-damaged sperm and a concomitant increase in the genomic instability of foetal cells and sperm chromatin abnormalities in F1 males, but this type of research is sparse and this topic deserves more attention [295–297].

9. Conclusions and future directions

The sperm comet assay is a well-established method for the assessment of sperm DNA fragmentation in various species. Nowadays, the sperm comet assay has its role in investigation of DNA-damaging effects in sperm of both animals and humans in various contexts - in environmental biomonitoring or the assessment of different physical and chemical agents *in vitro,* but also in studies of animal and human reproduction as well as reproductive and regulatory toxicology. In the context of reproductive toxicology, the sperm comet assay has been performed to test different sperm freezing protocols in order to minimize the effect of cryopreservation in the clinical setting. The assay has potential in the assessment of sperm DNA breakage, which could be of special significance for human reproduction in the context of natural and assisted fertilisation and understanding male infertility. One of the major problems with the sperm comet assay is the excessive inter-laboratory variability and use of a variety of different protocols. Therefore, attempts to standardize and harmonize these protocols should be undertaken to enable a direct comparison of results and to establish the sperm comet assay as a valid diagnostic tool. This could be done by developing guidelines at least for the common steps in the comet assay procedure, and adherence to such guidelines should be encouraged. Additionally, studies assessing the impact of variation in specific steps of the procedure are still needed in order to decrease the variation between experiments as well as between different laboratories. There are currently activities to establish the ploidy issue as a new approach in regulatory toxicology, involving the analysis of DNA damage in testicular cell suspensions on the basis of stage specific ploidy during spermatogenesis [298]. For somatic cells, the Organization for Economic Co-operation and Development adopted a comet assay guideline (OECD Test Guideline 489) for *in vivo* testing of DNA damage in animals [299], but still no OECD guidelines exist for *in vitro* genotoxicity testing. Possible reason for this is a lack of comprehension of the molecular mechanisms of comet formation. Another point is that distinguishing between detecting direct and indirect genotoxicity is a challenge with the standard *in vitro* comet assay. On the other hand, modifications of the assay may provide new insights and in that way need further attention [300]. Several issues related to the comet assay specificity, sensitivity as well as its limitations still need to be addressed before the assay can be accepted within a regulatory framework; hence, interlaboratory studies and future validation are still needed. This is in particular relevant for the sperm comet assay. Although a current literature search suggests that the sperm comet assay has in recent years been used more frequently, there is still a lack of specific knowledge on its significance for clinicians as well as for regulatory bodies. The long-term impact of sperm DNA damage on offspring's health is understudied and deserves further attention. To this end, the comet assay can be applied as a useful tool to study trans-generational effects of genotoxic exposures in animals and humans.

Declaration of Competing Interest

The authors report no declarations of interest.

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