Section I: Genesis of Comet Assay

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CHAPTER 1

The Comet Assay: A Versatile Tool for Assessing DNA Damage

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1.1 Introduction

Toxic substances and newer chemicals being added each year into the environment have led to increasing pollution of ecosystems as well as deterioration of air, water and soil quality. Excessive agricultural and industrial activities also adversely affect biodiversity, threatening the survival of species in a particular habitat as well as posing disease risks to humans. Some of the chemicals, *e.g.* pesticides and heavy metals, may cause deleterious effects in somatic or germ cells of the sentinel species as well as non-target species. Hazard prediction and risk assessment of chemicals, therefore, becomes imperative for assessing the genotoxic potential of chemicals before their release into the environment or for commercial use as well as to evaluate DNA damage in flora and fauna affected by contaminated or polluted

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habitats. The Comet assay has been widely accepted as a simple, sensitive and rapid tool for assessing DNA damage and repair in individual eukaryotic as well as some prokaryotic cells, and it has increasingly found application in diverse fields ranging from genetic toxicology to human epidemiology.

This review is an attempt to comprehensively examine the use of the Comet assay in diverse cell types from bacteria to humans, to assess the DNA-damaging potential of chemicals and/or environmental conditions. Sentinel species or bioindicator organisms in a particular ecosystem are the first to be affected by adverse changes in their environment. Determination of DNA damage in these organisms provides information about the genotoxic potential of their habitat at an early stage. This would allow for intervention strategies to be implemented for prevention or reduction of deleterious health effects in the sentinel species as well as in humans.

Ostling and Johanson[1] (in 1984) were the first to quantify DNA double stranded breaks in cells using a microgel electrophoresis technique, known as the single cell gel electrophoresis (SCGE) or Comet assay. Later, the assay was adapted by Singh *et al.*,[2] using alkaline conditions, which could assess both double- and single-strand DNA breaks as well as alkali-labile sites expressed as frank strand breaks in the DNA. Since its inception, the assay has been modified at various steps (cell isolation, lysis, electrophoresis, staining) to make it suitable for detecting various kinds of damage in different cells.[3,4] The assay is, now, a well established, simple, versatile, rapid, visual, and a sensitive, extensively used tool to assess DNA damage and repair, quantitatively as well qualitatively in individual cell populations.[5] Some other lesions of DNA damage such as DNA crosslinking (*e.g.* thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the Comet assay. It has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies,[3] genotoxicity testing[6] and human biomonitoring.[7,8] The field of ecotoxicology also provides a potential for use of Comet assay in natural ecosystems and has recently been reviewed to include the common experimental models used for studies, developments and/or modifications in protocols and improvements for future tests.[9]

Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution and the micronucleus assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 10^{10} Daltons of DNA), requirement for small number of cells (~10 000) per sample, flexibility to use proliferating as well as non-proliferating cells, low cost, ease of application and the short time needed to complete a study. It can be conducted on cells that are the first site of contact with mutagenic/carcinogenic substances (*e.g.* oral and nasal mucosal cells). The data generated at the single-cell level allows for robust types of statistical analysis.

A limitation of the Comet assay is that aneugenic effects,[10] and epigenetic mechanisms of indirect DNA damage such as effects on cell-cycle checkpoints are not detected. The other drawbacks such as single-cell data (which

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may be rate limiting), small cell sample (leading to sample bias), technical variability and interpretation are some of its disadvantages. However, its advantages far outnumber the disadvantages and hence it has been widely used in fields ranging from molecular epidemiology to genetic toxicology.

The present review deals with various models ranging from bacteria to humans, used in the Comet assay for assessing DNA damage (Figure 1.1).

1.1.1 Bacteria

Singh *et al.*[11] first used the Comet assay to assess the genetic damage in bacteria treated with 12.5–100 rad of X-rays. In the study, DNA double-strand breaks in the single electrostretched DNA molecule of *Escherichia coli* JM101 were determined using the neutral Comet assay. A significant increase in DNA breaks was induced by a dose as low as 25 rad, which was directly correlated to X-ray dosage (Table 1.1). The study supported the hypothesis that the strands of the electrostretched human DNA in the Comet assay represented individual chromosomes.

A modified version of the above Comet assay was used to assess the genotoxicity of antibacterial clay mineral mixture (CB) in *Escherichia coli.* CB leachate caused a significant increase in the double strand breaks in the bacterial cells, showing antimicrobial-mediated genotoxicity and suggesting the use of CB as an alternative bactericidal therapeutic.[12]

1.2 Plant Models

Plant bioassays are important tests which help detect genotoxic contamination in the environment. Plant systems can provide information about a wide range of genetic damage, including gene mutations and chromosome aberrations. Genotoxicity assessment in roots of plants like *Vicia faba*, *Nicotiana* and *Allium cepa*, have been widely conducted.[14,15] However, during the last decade, the plant Comet assay has been extensively applied to plants (leaves, shoots and roots) to detect DNA damage arising due to chemicals, radiation and heavy metals in polluted soil and comprehensively reviewed[16] (Table 1.1).

1.2.1 The Comet Assay in Lower Plants and Fungi

1.2.1.1 Fungi

Schizosaccharomyces pombe has been used as a model organism to investigate DNA damage due to chlorinated disinfectant, alum and polymeric coagulant mixture in drinking water samples.[39] The authors observed significantly higher ($P < 0.001$) DNA damage in chlorinated water (*i.e.* tap water) when compared with untreated (negative control) or distilled water (laboratory control). Hahn and Hock[40] used mycelia of *Sordaria macrospora* grown and treated with a variety of DNA-damaging agents directly on agarose

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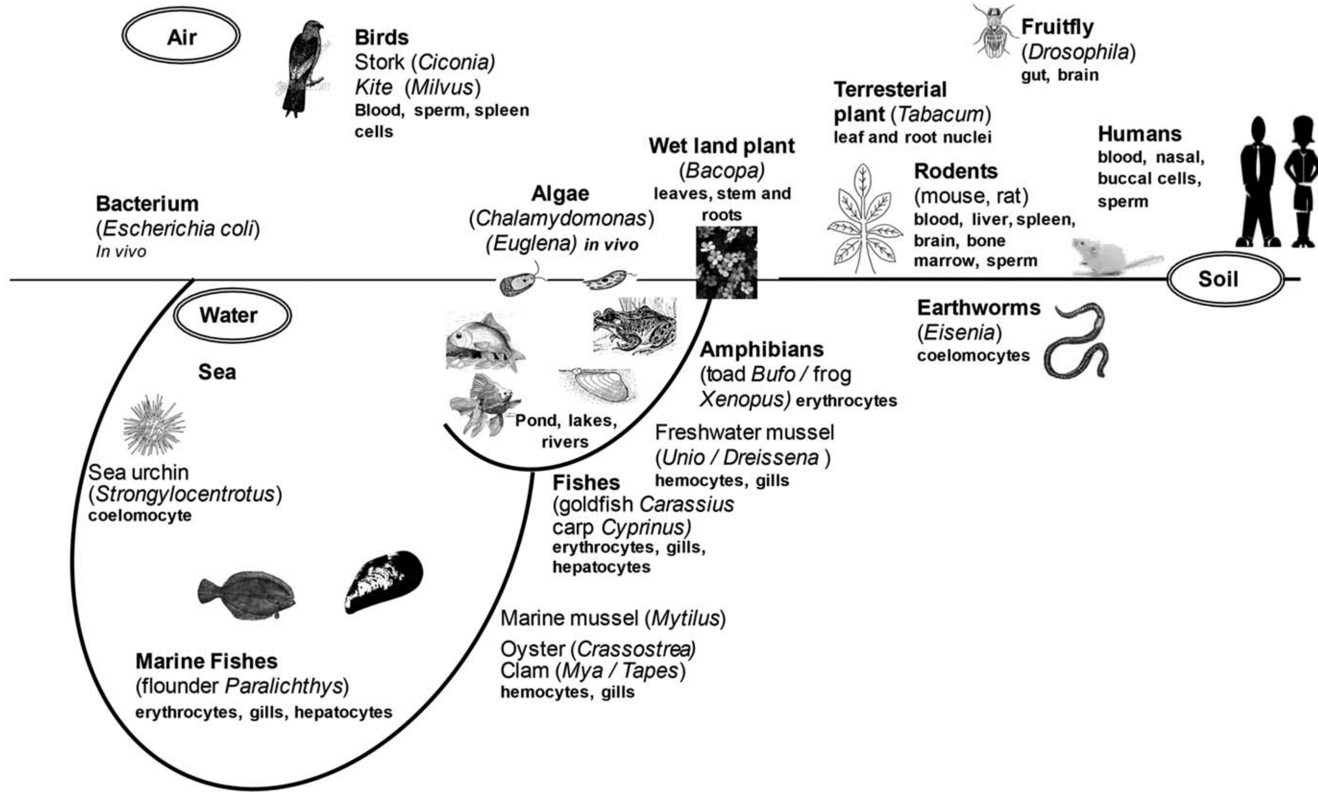

Figure 1.1 Schematic diagram of the use of comet assay in assessing DNA damage in different models from bacteria to humans. Reproduced from A. Dhawan, Comet assay: a reliable tool for the assessment of DNA damage in different models, *Cell Biol. Toxicol.*, 2009, **25**(1), 5–32, © Springer Science + Business Media B.V. 2008. With permission of Springer.

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Table 1.1 Comet assay for assessment of DNA damage—Bacteria and plants.

Model	Agent tested	Cells used	DNA damage[a]	Ref.
Bacteria				
Escherichia coli JM101	X-rays	Whole organism *in vivo*	↑	11
	Clay mineral mixture (CB)	Whole organism *in vivo*	↑	12
	Engineered nanoparticles	Whole organism *in vivo*	↑	13
Plant models				
Saccharomyces cerevisiae	Engineered nanoparticles	Whole organism *in vivo*	↑	13
	Cr(III)-citrate	Whole organism *in vivo*	↑	17
	Amaranth, Allura red azo dyes	Whole organism *in vivo*	↑	18
	Food additives	Whole organism *in vivo*	↑	19
Euglena gracilis	Organic pollutants	Whole organism *in vivo*	↑	20
Chlamydomonas reinhardtii	Chrysoidine	Whole organism *in vivo*	↑	21
	Paraquat herbicide	Whole organism *in vivo*	↑	22
Rhodomonas	UV (UVA and UVB) radiation	Whole organism *in vivo*	↑	23
Vicia faba	Arsenic	Root tip meristematic cells	↑	24
	Lead	Root tip meristematic cells	↑	25
	Organic pollutant	Root tip meristematic cells	↑	26
Tobacco (*Nicotiana tabacum*)	Ethyl methanesulphonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU), maleic hydrazide (MH).	Whole roots *in vivo*	↑	27
	o-Phenylenediamine (*o*-PDA), hydrogen peroxide and ethyl methanesulphonate (EMS)	Isolated root nuclei	–	28
	Heavy metal (Cd, Cu, Pb and Zn)	Leaf nuclei	↑	29
	Polychlorinated biphenyls	Leaf nuclei	↑	30
	Urban air pollutants	Leaf nuclei	↑	31
	TiO_2 nanoparticles	Leaf nuclei	↑	32
Potato plants (*Solanum tuberosum* var. *Korela*)	Heavy metal (Cd, Cu, Pb and Zn)	Nuclei from leaf tissue	↑	29
	Potato virus	Nuclei from leaf tissue	↑	33
Castor beans (*Ricinus communis*)	Air pollution	Leaf cells	Slight ↑	34
Phaeseolus vulgaris	Uranium	Root or shoot cells	–	35
Pisum sativum	Cr(VI)	Roots and leaves	↑	36
Bacopa monnieri L.	Ethyl methanesulphonate, methyl methanesulphonate Cadmium	Nuclei isolated from roots and leaves	↑ dose- and time-dependent roots > leaves	37
Duckweed (*Lemna*)	Industrial waste water	Leaves	↑	38

[a]↑ Significant increase in DNA damage; – no DNA damage reported. Data from A. Dhawan, *Cell Biol. Toxicol.*, 2009, **25**(1), 5–32.

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minigels for the assessment of genotoxicity using the Comet assay. This model allowed for the rapid and sensitive detection of DNA damage by a number of chemicals simultaneously. Few studies of the Comet assay in *Saccharomyces cerevisiae* have been reported, possibly due to the presence of the cell wall and the small amount of cellular DNA, however, it has been optimized as a model system to study oxidative DNA damage and repair,[41,42] as well as genotoxicity of chemicals[13,17,18] and food additives.[19]

1.2.1.2 Algae

Algae are aquatic unicellular plants, which provide information regarding the potential genotoxicity of the water in which they grow. Being single-celled organisms, they can be used as a model for risk assessment monitoring of environmental pollution of aquatic environments using the Comet assay. The freshwater green algae species, *Pseudokirchneriella subcapitata* and *Nannocloris oculata* revealed DNA damage by the insecticide Chlorpyriphos and fungicide Tebuconazole at low concentrations.[43] The unicellular green alga *Chlamydomonas reinhardtii* has shown DNA damage due to known genotoxic chemicals[21,44] and the herbicide paraquat[22] and also demonstrated that oxidative stress was better managed by the algal cells under light than dark conditions.[44] The Comet assay successfully evaluated chemically-induced DNA damage and repair in *Euglena gracilis* and the responses were found to be more sensitive than those of human lymphocytes under the same treatment conditions.[45] The ease of culturing and handling *E. gracilis* as well as its sensitivity makes it a useful tool for testing the genotoxicity of chemicals and monitoring environmental pollution and it can be used as a part of bioassay for ecotoxicology studies. *E. gracilis* demonstrated increased genotoxicity in Comet assay parameters due to organic extracts from Taihu Lake (China), and has thus been selected as a bioindicator organism to provide early warning of organic pollutants.[20] A modified version of the Comet assay was used as an alternative technique to assess DNA damage due to UV radiation in *Rhodomonas* sp. (Cryptophyta), a marine unicellular flagellate.[23]

1.2.2 The Comet Assay in Higher Plants

Recently there has been an increase in the use of the Comet assay in higher plants to study DNA damage and repair, to understand the effects of genotoxicity of pollutants and the environment. The effect of various stressors on DNA damage in plants, the correlation of the DNA damage with cellular responses[16] and DNA repair[46,47] have been reviewed and recommendations regarding the method have also been made for increasing the reliability and throughput of the Comet assay in plants.[48]

Vicia faba has been widely used for the assessment of DNA damage using the Comet assay. Strand breaks and abasic (AP) sites in meristematic nuclei of *V. faba* root tips were studied by the neutral and alkaline Comet assay.[49,50]

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The alkaline electrophoresis procedure was found to be most sensitive at low doses, while the neutral electrophoresis procedure yielded an optimal dose–response curve within a wider dose range. Angelis *et al.*[49] also suggested that the Comet assay was able to detect a phenomenon resembling clastogenic adaptation at molecular level. *Vicia faba* used as a bioindicator plant has shown increased DNA damage due to inorganic arsenic in water (correlated with abnormal molecular changes at 20 and 30 mg l^{-1} concentration),[24] lead (due to oxidative stress at 10 μM concentration),[25] and persistent organic pollutant-containing agricultural soils from Tlaxcala, Mexico.[26]

Gichner and Plewa[51] developed a sensitive method for isolation of nuclei from leaf tissue of *Nicotiana tabacum*, which, due to its high resolution and constant low tail moment values for negative controls, could be incorporated in *in situ* plant environmental monitoring.[51] The Comet assay has been used to study the effect of alkylating agents in tobacco seedlings.[52] A small but significant increase in DNA damage compared with controls was noted in heterozygous tobacco and potato plants grown on soil contaminated with heavy metals.[29] The tobacco and potato plants with increased DNA damage were also found to be severely injured (inhibited growth, distorted leaves), which may be associated with necrotic or apoptotic DNA fragmentation. Detection of concentration-dependent genotoxicity of urban air pollutants in leaf nuclei[31] and titanium dioxide (TiO_2) nanoparticles,[32] in *Nicotiana* using the Comet assay has shown it to be useful for environmental monitoring.

No DNA damage was observed in the root or shoot cells of *Phaeseolus vulgaris* treated with different concentrations of uranium.[35] Cr(VI) showed concentration-dependent increases in DNA damage as detected by Comet assay and complemented by flow cytometry in leaves and roots of *Pisum sativum*, revealing clastogenic action of chromium.[36] The alkaline Comet assay was used to measure DNA damage and repair in the model plant *Arabidopsis* and rye grass exposed to X-rays.[47] Rapid and slow phases of repair were observed for acute exposures of 5 and 15 Gy, and a possible explanation of homologous repair (HR) of double-strand breaks during the slow phase was proposed.[47] For the first time Comet–fluorescence *in situ* hybridization (FISH) was conducted in the model plant species *Crepis capillaris* following exposure of seedlings to maleic hydrazide (MH), demonstrating 5S rDNA in the tail of the Comets, and suggesting Comet–FISH as a tool for environmental monitoring.[53]

The major drawback with plant models was the fact that exposure needs to be given through the soil and it is difficult to say whether the result demonstrates synergies with other chemicals in the soil or non-availability of the toxicant due to its soil binding affinity. To circumvent this disadvantage, Vajpayee *et al.*,[37] used *Bacopa monnieri* L., a wetland plant, as a model for the assessment of ecogenotoxicity using the Comet assay. *In vivo* exposure to cadmium (0.01–500 μM) for 2, 4 and 18 h resulted in dose- and time-dependent increases in DNA damage in the isolated roots and leaf nuclei, with roots showing greater DNA damage than leaves. *In vitro* (acellular) exposure of nuclei from leaves of *B. monnieri* to 0.001–200 μM cadmium

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resulted in significant (P <0.05) levels of DNA damage. Another bioindicator plant duckweed (*Lemna*) was used to study effects of industrial wastewater samples from environmental monitoring sites along the river Sava (Croatia) and showed a marked increase in DNA damage.[38]

Reviews of the use of Comet assay in higher plants have been recently published which discuss protocols and its use in environmental genotoxicity research,[54] as well as applications in DNA repair studies and mutation breeding.[55] These studies revealed that DNA damage measured in plants using the Comet assay is a good model for *in situ* monitoring and screening of genotoxicity of polluted environments. Higher plants can also be used as an alternative first-tier assay system for the detection of possible genetic damage resulting from polluted waters or effluents due to industrial activity or agricultural run offs.

1.3 Animal Models

Animal models have long been used to assess the safety or toxicity of chemicals and finished products. With the advancements in technology, use of knockouts and transgenic models has become common for mimicking the effects in humans. The Comet assay has globally been used for assessment of DNA damage in various animal models.

1.3.1 Lower Animals

The Comet assay has been used in a unicellular protozoan and invertebrates for establishing the safety of the environment in which these species are found (Table 1.2)

1.3.1.1 Protozoan

Tetrahymena thermophila is a unique unicellular protozoan, with both somatic and germ nucleus present in the same cell, and is widely used for genetic studies due to its well characterized genome. Therefore it was validated as a model organism for assessing DNA damage using a modified Comet assay protocol standardized with known mutagens such as phenol, hydrogen peroxide and formaldehyde.[56] The method was then used for the assessment of genotoxic potential of influent and effluent water samples from a local municipal wastewater treatment plant.[56] The method provided an excellent, low level detection of genotoxicants and proved to be a cost-effective and reliable tool for genotoxicity screening of waste water. Ecological risk assessment of the organic pollutant dechlorane plus (DP) was conducted in *Tetrahymena* using the Comet assay, which showed its potential genotoxicity at high levels.[57] Melamine was found to be highly toxic to the *Tetrahymena* genome which also caused apoptosis.[58] An acellular Comet assay in *Tetrahymena* has also been used to study the genotoxicity of TiO_2 nanoparticles.[59]

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1.3.1.2 Invertebrates

Various aquatic (marine and freshwater) and terrestrial invertebrates have been used for genotoxicity studies employing the Comet assay (Table 1.2) which have also been reviewed.[9,93,125,126] Cells from haemolymph, embryos, gills, digestive glands and coelomocytes from mussels (*Mytilus edulis*), zebra mussel (*Dreissena polymorpha*), clams (*Mya arenaria*) and polychaetes (*Nereis virens*), have been used for ecogenotoxicity studies using the Comet assay. DNA damage has also been assessed in earthworms and fruit fly (*Drosophila*). The Comet assay has been employed to assess the extent of DNA damage at polluted sites in comparison to reference sites in the environment and, in the laboratory, it has been used as a mechanistic tool to determine pollutant effects and mechanisms of DNA damage.[78]

1.3.1.2.1 The Comet Assay in Mussels. Adverse effects of contaminants in the aquatic environment have been studied in freshwater and marine mussels as they are important pollution indicator organisms. These sentinel species provide the potential for environmental biomonitoring of aquatic environments which they inhabit. The Comet assay in mussels can be used to detect a reduction in water quality caused by chemical pollution.[75,127] *Mytilus edulis* has been widely used for Comet assay studies to evaluate DNA strand breaks in gill and digestive gland nuclei due to polycyclic aromatic hydrocarbons (PAHs) including benzo[*a*]pyrene (B[*a*]P),[70] and oil spills with petroleum hydrocarbons.[92] However, the damage returned to normal levels, after continued exposure to high dose (20 ppb-exposed diet) of B[*a*]P for 14 days. This was attributed to an adaptive response in mussels to prevent the adverse effects of DNA damage.[70] Repairable DNA damage with B[*a*]P was also observed with *Mytilus galloprovincialis* and the green lipped mussels (*Perna viridis*).[85] Effects of ionizing radiation, due to anthropogenic addition of radionuclides in aquatic environment, have been found to alter DNA damage and RAD 1 genes in *Mytilus* tissues.[73] Since the biomonitoring of the indicator organisms *in situ* may cause time constraints and not all samples may be processed at the same time, the cryopreservation of samples for later analysis in laboratory would be beneficial. Kwok *et al.*[128] used different media for this study and found that preserved haemocytes samples of *Mytilus* may be stored at cryogenic temperatures for a month without change in DNA damage for analysis in Comet assay.[128]

Inter-individual variability, including seasonal variations in DNA damage have been reported from some studies, both in laboratory and field,[71,130,131] hence baseline monitoring has to be carried out over long time intervals. Haemocytes of freshwater Zebra mussel *Dreissena polymorpha* have shown temperature-dependent DNA damage showing that the mussels are sensitive to changes in water temperatures,[64] and monitoring ecogenotoxicity with these species should account for variations in temperatures. The Comet assay in haemocytes of *D. polymorpha* was used as a

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Table 1.2 Comet assay for assessment of DNA damage—Animal models (Invertebrates).

Model	Agent tested	Cell used	DNA damage[a]	Ref.
Tetrahymena thermophila	Phenol, hydrogen peroxide and formaldehyde, influent and effluent water samples	Whole animal *in vivo*	↑	56
	Dechlorane plus (DP)	Whole animal *in vivo*	↑	57
	Melamine	Whole animal *in vivo*	↑	58
	Titanium dioxide nanoparticles	Acellular	↑	59
Invertebrates bivalves				
Freshwater bivalve zebra mussel (*Dreissena polymorpha)*	Polybrominated diphenyl ethers (PBDEs)	Haemocytes	↑↑	60
	Sodium hypochlorite and chlorine dioxide) and peracetic acid	Haemocytes	↑	61
	NSAIDS (diclofenac, ibuprofen and paracetamol)	Haemocytes	↑	62
	Pentachlorophenol	Haemocytes	↑	63
	Varying temperatures	Haemocytes	↑	64
	Polluted waters	Haemocytes	↑	65
Mytilus edulis	Cadmium (Cd)	Gills	–	66
	Styrene	Haemolymph cells	↑	67
	Tritium	Haemocytes	↑	68
	Marine waters (Denmark), French Atlantic Coast	Haemocytes	↑	69
	Polycyclic aromatic hydrocarbons	Gill and haemolymph	↑	70
	Seasonal variation	Gill and haemocytes	↑	71
	C60 fullerene and fluoranthene	Haemocytes	Concentration-dependent ↑ alone and ↑↑ together	72
	Ionizing radiation	Haemocytes	↑	73
	Tamar estuary waters (England)	Haemocytes	↑ at site of high Cr concentration	74
Mytilus galloprovincialis	Environmental stress	Haemocytes	↑	75
	Copper oxide and silver nanoparticles	Haemolymph cells	↑	76
	Titanium dioxide nanoparticles	Haemocytes	↑	77
Freshwater mussels				
Unio tumidus	Polyphenols	Digestive gland cells	↑	78, 79
	Base analogue 5-Fluorouracil (FU)	Haemocytes	↑	80

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Unio pictorum	Base analogue 5-Fluorouracil (FU)	Haemocytes	↑	80
Golden mussel (*Limnoperna fortunei*)	Guaíba Basin water	Haemocytes	↑	81
Bivalve mollusc (*Scapharca inaequivalvis*)	Organotin compounds (MBTC, DBTC and TBTC)	Erythrocytes	↑	82
Vent mussels (*Bathymodiolus azoricus*)	Hydrostatic pressure change	Haemocytes and gill tissues	↑	83, 84
Green-lipped mussels				
Perna viridis	Benzo[*a*]pyrene	Haemocytes	↑	85
Perna canaliculus	Cadmium	Haemocytes	↑	86
Freshwater mussel (*Utterbackia imbecillis*)	Chemicals used in lawn care (atrazine, glyphosate, carbaryl and copper)	Glochidia	↑	87
Oyster (*Crassostrea gigas*)	Cryopreservation	Spermatozoa	↑	88
	Diuron (0.05 $\mu g\ l^{-1}$), glyphosate	Spermatozoa	↑, –	89
Manila clam (*Tapes semidecussatus*)	Sediment-bound contaminants	Haemolymph, gill and digestive gland	↑	90, 91
Clams				
Mya arenaria	Petroleum hydrocarbons	Haemocytes and digestive gland cells	–	92
Ruditapes decussatus	PAH	Gills	↑	93
Earthworms				
Eisenia foetida	Soil from industrialized contaminated areas	Coelomocytes	↑	94
	Sediment from polluted river	Coelomocytes	↑	95
	Waste water irrigated soil	Coelomocytes	↑	96
	Commercial parathion	Sperm cells	↑	97
	Imidacloprid and RH-5849	Coelomocytes	↑	98
	PAH contaminated soil and hydrogen peroxide, Cadmium (*in vitro*)	Eleocytes	↑	99
	Nickel chloride	Coelomocytes	↑	100
	Dechlorane plus	Coelomocytes and Spermatogenic cells	↑	101
	Ionizing radiation (*in vivo* and *in vitro*)	Coelomocytes	↑	102
	Radiation and mercury	Coelomocytes	↑ synergistic effect	103
	Nickel and deltamethrin, with humic acid	Coelomocytes	↑, synergistic effect, damage ↓ with humic acid	104

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Table 1.2 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
	Lead and BDE209	Coelomocytes	↑ alone, antagonistic effect	105
Eisenia hortensis	Cobalt chloride	Coelomocytes	↑ dose-dependant	106
Aporrectodea longa (Ude)	Soil samples spiked with benzo[*a*]pyrene (B[*a*]P) and/or lindane	Intestine and crop or gizzard cells	↑ intestine > crop	107
Other invertebrates				
Fruit fly (*Drosophila melanogaster*)	Ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), *N*-ethyl-*N*-nitrosourea (ENU) and cyclophosphamide (CP)	Gut and brain cells of first instar larvae	↑	108, 109
	Cypermethrin	Brain and anterior midgut cells	↑	110
	Leachates of industrial waste	Gut and brain cells of first instar larvae	↑	108
	Cisplatin	Midgut cells	↑	111
	Hexavalent chromium	Larval haemocytes	↑↑	112
	Zinc oxide nanoparticles	Larval haemocytes	↑ at high dose.	113
	Copper oxide nanoparticles,	Larval haemocytes	↑	114
	Cadmium selenium (CdSe) quantum dots	Larval haemocytes	↑	115
Grasshoppers (*Chorthippus brunneus*)	Different polluted sites	Larval brain cells	↑↑ in heavy polluted site	116
	Paraquat (*in vitro*, *in vivo*)	Larval brain cells	↑ time dependent	117
Sea urchins (*Strongylocentrotus droebachiensis*)	Dispersed crude oil	Coelomocytes	↑ concentration-dependent	118
Grass shrimp, (*Paleomonetes pugio*)	UV, benzo[*a*]pyrene, and cadmium	Embryos	↑ damage and decreased repair	119
	Estuarine sediments	Hepatopancreas	↑	120
	Coal combustion residues	Hepatopancreas	↑	121
Sea anemone (*Anthopleura elegantissima*)	Hydrogen peroxide ethylmethanesulphonate (EMS) or benzo[*a*]pyrene (B[*a*]P)	Blood cells	↑ dose response	122
Marine invertebrate (*Donax faba*)	Pesticide Chlorpyriphos and fungicide Carbendazime	Gill, body and foot cells	↑	123
Polychaete (*Nereis diversicolor*)	Nano-, micro- and ionic-Ag	Coelomocytes	↑↑ Nano >micro >ionic	124

[a]↑ Significant increase in DNA damage, ↑↑ highly significant increase in DNA damage; ↓ decrease in DNA damage; – no DNA damage reported.

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tool in determining the potential genotoxicity of water pollutants,[60–63] and Klobucar *et al.*[65] suggested that haemocytes from caged, non-indigenous mussels could be used for Comet assay for monitoring genotoxicity of freshwater. The hOGG1 enzyme was used in the Comet assay to evaluate 8-oxo-2′-deoxyguanosine (8-oxo-dG) as a marker of oxidative DNA damage in *D. polymorpha*.[129]

DNA damage and repair studies in vent mussels, *Bathymodiolus azoricus*, have been carried out to study the genotoxicity of naturally contaminated deep-sea environment.[83,84] The vent mussels demonstrated similar sensitivity to environmental mutagens to that of coastal mussels and thus could be used for ecogenotoxicity studies of deep sea waters using the Comet assay. Villela *et al.*[132] used the golden mussel (*Limnoperna fortunei*) as a potential indicator organism for freshwater ecosystems due to its sensitivity to water contaminants.

In vitro Comet assay has also been used in cells of mussels, which can be used to screen genotoxic agents destined for release or disposal into the marine environment. Dose-responsive increases in DNA strand breakages were recorded in digestive gland cells[133] haemocytes[134] and gill cells[134] of *M. edulis* exposed to both direct-acting (hydrogen peroxide and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[*5H*]-furanone) and indirect-acting (B[*a*]P, 1-nitropyrene, nitrofurantoin and *N*-nitrosodimethylamine) genotoxicants. Digestive gland cells[78,135] and haemocytes[80] of *Unio tumidus* were also used for *in vitro* studies of DNA damage and repair by different compounds.

1.3.1.4 The Comet Assay in Other Bivalves

Coughlan *et al.*[90] showed that the Comet assay could be used as a tool for the detection of DNA damage in clams (*Tapes semidecussatus*) as biomonitor organisms for sediments. Significant DNA strand breaks were observed in cells isolated from haemolymph, gill and digestive gland from clams exposed to polluted sediment.[90,91] Comet assay was used for the assessment of sperm DNA quality of cryopreserved semen in Pacific oyster (*Crassostrea gigas*) as it is widely used for artificial fertilization.[88] The Comet–FISH assay, conducted in haemocytes of *C. gigas*, was shown to have potential for detecting DNA damage of target genes, induced by toxicant exposure and to allow better understanding of the impact of genotoxicity on animal physiology and fitness.[136] Gielazyn *et al.*[137] demonstrated the use of lesion-specific DNA repair enzyme formamidopyrimidine glycosylase (Fpg) to enhance the usefulness and sensitivity of the Comet assay in studying oxidative DNA damage in isolated haemocytes from oyster (*Crassostrea virginica*) and clam (*Mercenaria mercenaria*). The herbicide diuron induced significant DNA damage in oyster spermatozoa at 0.05 μg l^{-1} upwards while its environmental concentrations significantly affected embryo–larval development, showing deleterious effects of herbicide in non-target organisms.[89]

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The Comet assay detecting DNA strand breaks has demonstrated that higher basal levels of DNA damage are observed in marine invertebrates, hence the protocol followed in these animals should be considered for biomonitoring the ecogenotoxicity of a region.[138]

1.3.1.5 The Comet Assay in Earthworms

The Comet assay applied to earthworms is a valuable tool for monitoring and detection of genotoxic compounds in terrestrial ecosystems[94–105] (Table 1.2). Since the worms feed on the soil they live in, they are a good indicator of the genotoxic potential of the contaminants present in the soil and thus used as a sentinel species.

Coelomocytes from *Eisenia foetida* have been used for biomonitoring purposes, to assess DNA damage in worms exposed to soil samples from industrialized contaminated areas[94] and sediment samples from polluted river systems.[95] Ecogenotoxicity studies have shown dose dependent DNA strand breaks caused by insecticide[97] and pesticides[98] in *E. foetida* as well as *Pheretima* species[139] demonstrating that pesticides could also have adverse effects on non-target species. Ionizing radiation affects the soil ecology, as it induced oxidative damage in spermatogenic cells of *E. foetida* and also reduced reproduction at dose rates at or >4 mGy h^{-1}.[102] Radiation with exposure to mercury produced synergistic effects and increased damage to DNA.[103] Humic acid was found to alleviate nickel- and deltamethrin-induced toxicity in earthworms, and could be used to reduce oxidative damage to DNA, lipids and proteins.[104] Medicinal therapy using peloids (natural mud), despite usually being beneficial, may also pose a risk of toxic effects as was seen in a study with *E. foetida* exposed to peloids.[140]

In vitro exposure of primary cultures of coelomocytes to nickel chloride as well as exposure of whole animals either in spiked artificial soil water or in spiked cattle manure substrates exhibited increased DNA strand breaks due to the heavy metal.[100] The eleocytes cells, a subset of coelomocytes produced increased DNA strand breaks under both *in vitro* and *in vivo* conditions and could be used a sensitive biomarker for genotoxicity in earthworms.[99] Another earthworm *Aporrectodea longa* (Ude), when exposed to soil samples spiked with B[*a*]P and/or lindane demonstrated genotoxicity in the intestinal cells to be more sensitive to the effect of the toxicants than the crop or gizzard cells.[107]

Fourie *et al.*[141] used five earthworm species (*Amynthas diffringens*, *Aporrectodea caliginosa*, *Dendrodrilus rubidus*, *Eisenia foetida* and *Microchaetus benhami*) to study genotoxicity of cadmium sulphate, with significant DNA damage being detected in *E. foetida* followed by *D. rubidus* and *A. caliginosa*. The study showed the difference in sensitivity of species present in an environment and its influence on the genotoxicity risk assessment. Hence for environmental biomonitoring, specific species have to be kept in mind to reduce false negative results.

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1.3.1.6 *The Comet Assay in* Drosophila

The simple genetics and developmental biology of *Drosophila melanogaster* has made it the most widely used insect model. It has been recommended as an alternate animal model by the European Centre for the Validation of Alternative Methods[142] and evolved into a model organism for toxicological studies.[143,144] *D. melanogaster* has been used as an *in vivo* model (Table 1.2) for assessment of genotoxicity[108–115] and oxidative DNA damage[145] as well as for *in vitro* studies[146] using the Comet assay. Cisplatin induced adducts in *D. melanogaster* are influenced by conditions of nucleotide excision repair, and this correlates well with DNA damage as seen in Comet assay.[147] Recently, the Comet assay in *Drosophila* as an *in vivo* model has been used to assess the genotoxicity of zinc, copper and cadmium nanomaterials, which have demonstrated oxidative DNA damage.[113–115]

The studies in *Drosophila* have shown it to be a good alternative to animal models for the assessment of *in vivo* genotoxicity of chemicals using the Comet assay.

1.3.1.7 The Comet Assay in Other Invertebrates

Nereis virensa, a polychaete, plays an important role in the distribution of pollutants in sediments due to its unique property of bioturbation. These worms are similar to earthworms in soil and can be used for genotoxicity assessment of sediments. They have been used to study sediment-associated toxicity of silver nanoparticles, and bioaccumulation in the body was also shown.[124] Genotoxicity of intracoelomically injected B[*a*]P was assessed in worm coelomocytes using Comet assay, however, *Nereis* species was not found to be suitable for assessing PAH genotoxicity due to their lack of metabolic capability to convert B[*a*]P to its toxic metabolite.[148]

DNA damage was assessed in neuroblast cells of brains of first instars of grasshoppers (*Chorthippus brunneus*) exposed to various doses of zinc from a polluted site, to understand the mechanism of toxicity in the insects due to industrial pollutants.[149] Comet assay parameters in brain cells of larvae originating from eggs of grasshoppers from different polluted sites have shown an association between increased DNA damage and heavy environmental pollution.[116] Paraquat caused increased DNA damage in brain cells in both *in vitro* and *in vivo* administrations.[117]

Chronic exposure to coal combustion residues from coal-fired electrical generation in estuarine grass shrimp, *Palaemonetes pugio*, caused DNA damage in hepatopancreatic cells of adult shrimps as compared with the reference shrimp as seen in the Comet assay.[121] The Comet assay in planarians is an important test for environmental monitoring studies since these are simple organisms with high sensitivity, low cost and a high proliferative rate.[150] The genotoxic potential of polluted waters from Diluvio's Basin, Norflurazon, a bleaching herbicide[151] and copper sulfate[152] was evaluated in planarians, where, significant increases in primary DNA damage were

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observed in these species. These studies have also demonstrated the use of the Comet assay in biomonitoring diverse environmental conditions utilizing sentinel species.

1.4 Higher Animals

1.4.1 Vertebrates

Studies of vertebrate species where the Comet assay is used have included fishes, amphibians, birds and mammals. Cells (blood, gills, kidneys and livers) of different fishes, tadpoles and adult frogs, as well as rodents have been used for assessing *in vivo* and *in vitro* genotoxicity of chemicals, and human biomonitoring has also been carried out employing the Comet assay (Table 1.3).

1.4.1.1 The Comet Assay in Fishes

Various fishes (freshwater and marine) have been used for environmental biomonitoring, as they are endemic organisms, which serve as sentinel species for a particular aquatic region, to the adverse effects of chemicals and environmental conditions. The Comet assay has found wide application as a simple and sensitive method for evaluating *in vivo* as well as *in vitro* DNA damage in different tissues (gills, liver and blood) of fishes exposed to various xenobiotics in the aquatic environment (Table 1.3).

The basal level of DNA damage detected in the Comet assay has been shown to be influenced by various factors, such as the temperature of water in erythrocytes of mullet and sea catfish,[156,157] age and gender in dab (*Limanda limanda*[179]), exhaustive exercise[154] and seasonal changes[155] in chub. Therefore, these factors should be accounted for during environmental biomonitoring studies. The high intra-individual variability may also affect the sensitivity of the assay.[179] The protocol and experimental conditions used for the Comet assay for monitoring marine ecosystems may lead to differences in the results obtained. Also, chemical and mechanical procedures to obtain cell suspensions may lead to additional DNA damage.[318] Anaesthesia did not contribute towards DNA damage *in vivo* in methyl-methanesulfonate (MMS)-treated fishes and the anaesthetic benzocaine did not alter the DNA damage in erythrocytes after *in vitro* exposure to MMS or H_2O_2.[319] Hence keeping in mind animal welfare, multi-sampling of the same fish can be conducted. Recently, nanomaterials toxicity has gained importance in aquatic toxicology as nanomaterials synthesis and use has increased. Its impact on the aquatic environment and on fishes needs to be elucidated and this calls for development and implementation of protocols for nanomaterial genotoxicity in ecotoxicology.[320–322]

In vitro studies on fish hepatocytes,[182,185] primary hepatocytes and gill cells[186] as well as established cell lines (with metabolic competence[189–191]) using the Comet assay have also been conducted to assess the genotoxicity of

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Table 1.3 Comet assay for assessment of DNA damage–Animal models (Vertebrates).

Model	Agent tested	Cell used	DNA damage[a]	Ref.
Fishes				
Chub (*Leuciscus cephalus*)	PAHs, PCBs, organochlorine pesticides (OCPs), and heavy metals	Hepatocytes	↑	153
	Exhaustive exercise	Erythrocytes	↑	154
	Seasonal change at polluted sites.	Gills, liver, blood	↑ in spring/autumn, gills and liver > blood	155
Estuarine mullet (*Mugil* sp.) and sea catfish (*Netuma* sp.)	Organochlorine pesticides and heavy metals	Erythrocytes	↑	156
	High temperature	Erythrocytes	↑	157
Fresh water teleost (*Mystus vittatus*)	Endosulfan	Gill, kidney and erythrocytes	↑ in all cells	158
Fresh water murrel (*Channa punctatus*)	Tannery effluent in Ganges, India	Gills	↑	159
Tilapia (*Oreochromis niloticus*)	Antibiotics Florfenicol (FLC) and oxytetracycline (OTC)	Blood erythrocytes	↑	160
Eastern mudminnow (*Umbra pygmaea* L.)	Rhine water for 11 days	Blood erythrocytes	↑	161
Neotropical fish *Prochilodus lineatus*	Diesel water soluble fraction acute (6, 24 and 96 h) and subchronic (15 days) exposures, Cypermethrin, *in vivo*	Erythrocytes	↑	162
	Ethyl methanesulfonate, hydrogen peroxide (*in vitro*)	Epithelial gill cells	↑ *in vivo* and *in vitro*	163
Freshwater goldfish (*Carassius auratus*)	Technical herbicide Roundup (glyphosate)	Erythrocytes	↑↑ dose-dependent	164
	ADDB and PBTA-6	Erythrocytes	↑ dose-dependent	165
Turbot (*Scophthalmus maximus* L.)	Sediment collected from polluted sites in Cork Harbour (Ireland)	Hepatocytes	↑	166
	PAH by different routes	Erythrocytes	↑ by all routes	167
Zebra fish (*Danio rerio*)	Methyl methanesulphate	Gill, gonads and liver cells	↑ in all cells	168
Brazilian flounder (*Paralichthys orbignyanus*)	Contaminated estuary waters	Blood cells	↑↑	169

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Table 1.3 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
European flounder (*Platichthys flesus*)	Different estuaries, seasons and genders	Blood cells	↑	170
Carp (*Cyprinus carpio*).	Disinfectants	Erythrocyte	↑	171
	NSAID-manufacturing plant effluent	Erythrocyte	↑	172
Armoured catfish (*Pterygoplichtys anisitsi*)	Diesel and biodiesel	Erythrocytes	↑	173
Trout (*Oncorhynchus mykiss*)	Cryopreservation (Freeze-thawing)	Spermatozoa	Slight ↑	174
European eel (*Anguilla anguilla*)	Benzo[*a*]pyrene, Arochlor 1254, 2-3-7-8-tetrachlorodibenzo-*p*-dioxin and beta-naphthoflavone	Erythrocytes	↑	175
	Herbicides-Roundup, Garlon	Erythrocytes	↑	176
Eelpout (*Zoarces viviparus*)	Oil spill (PAH)	Nucleated erythrocytes	↑	177
Gilthead sea bream (*Sparus aurata*)	Copper	Erythrocytes	↑↑	178
Dab (*Limanda limanda*)	PAHs and PCBs polluted waters of English channel Gender and age	Blood cells	↑ in adults and males	179
Hornyhead turbot (*Pleuronichthys verticalis*)	Sediments collected from a natural petroleum seep (pahs)	Liver cells	↑	180
In vitro				
Carp (*Cyprius carpio*)	Organic sediment extracts from the North Sea (Scotland)	Leukocytes	↑	181
Trout (*Oncorhynchus mykiss*)	Cadmium	Hepatocytes	↑	182
	Tannins	Erythrocytes	↓	183
	Diaryl tellurides and ebselen (organoselenium)	Erythrocytes	↓	184
	Oil sands processed water, (PAH and naphthnic acids)	Hepatocytes (*in vitro*)	↑	185
Zebrafish (*Danio rerio*)	Surface waters of German rivers, Rhine and Elbe	Hepatocytes and gill cells	↑	186
Danio rerio (ZFL) hepatocyte cell line	Biodiesel	Hepatocytes	↑	187
Rainbow trout hepatoma cell line (RTH-149)	Water samples from the polluted Kishon river (Israel)	Liver	↑	188

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Rainbow trout gonad (RTG-2) cell line	4-nitroquinoline-*N*-oxide *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, benzo[*a*]pyrene, nitrofurantoin, 2-acetylaminofluorene, dimethylnitrosamine, and surface waters	Gonad	↑ dose dependent response	189
Rainbow trout liver (RTL-W1) cell line	2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	Epitheloid liver	Slight ↑	190
	Coal tar run off water	Epitheloid liver	↑	191
Amphibians				
Amphibian larvae (*Xenopus laevis and Pleurodeles waltl*)	Cadmium ($CdCl_2$)	Erythrocytes	↑ concentration and time dependent	192
	Captan (*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide)	Erythrocytes	↑ concentration and time dependent	193
Amphibian larva (*Xenopus laevis*)	Benzo[*a*]pyrene, ethyl and methyl methanesulfonate	Erythrocytes	–	194
	Aqueous extracts of five sediments from French channels	Erythrocytes	↑	195
Toad (*Bufo raddei*)	Petrochemical (mainly oil and phenol) polluted area	Liver cells and erythrocytes	↑	196
Southern toad (*Anaxyrus terristris*)	Low-dose-rate ionizing radiation	Red blood cells	↓ at ≥21 mGy	197
Toad (*Xenopus laevis*, and *Xenopus tropicalis*)	Bleomycin induced DNA damage and repair	Splenic lymphocytes	↑ DNA damage in *X. tropicalis* > *X. laevis*	198
Xenopus laevis, and *Xenopus tropicalis*			DNA repair in *X. laevis* > *X. tropicalis*	
Tadpoles of *Rana N. Hallowell*	Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine] and RH-5849 [2′-benzoyl-l′-*tert*-butylbenzoylhydrazinel]	Erythrocytes	↑	199
Tadpoles (*Rana hexadactyla*)	Sulfur dyes (Sandopel Basic Black BHLN, Negrosine, Dermapel Black FNI, and Turquoise Blue) used in the textile and tannery industries	Erythrocytes	↑↑	200
Tadpoles of Bullfrog (*Rana catesbeiana*)	Herbicides AAtrex Nine-O (atrazine), Dual-960E (metalochlor), Roundup (glyphosate), Sencor-500F (metribuzin), and Amsol (2,4-D amine)	Erythrocytes	↑↑	201

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Table 1.3 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
Tadpole *Rana clamitans* *Rana pipiens*	Agricultural regions Industrial regions	Erythrocytes	↑ industrial regions > agricultural regions	202
Tadpoles (*Rana limnocharis*)	Cadmium ($CdCl_2$)	Erythrocytes	↑	203
	Sodium arsenite	Whole blood	↑	204
Eurasian marsh frog (*Pelophylax ridibundus*)	Pollution in the different lakes in central Anatolia, Turkey.	Blood cells	↑	205
Anuran amphibian (*Hypsiboas faber*)	Heavy metal, in coal open-cast mine	Blood cells	↑	206
Frog tadpoles (*Dendropsophus minutes*)	Agrochemicals	Blood cells	↑	207
In vitro				
Xenopus laevis	high peak-power pulsed electromagnetic field	Erythrocytes	↑ due to rise in temperature	208
Birds				
Wild nestling white storks (*Ciconia ciconia*)	Heavy metals and arsenic	Blood cells	↑ correlated with arsenic	209
	Toxic acid mining waste rich in heavy metals	Blood cells	↑↑	210–212
Black kites (*Milvus migrans*)	Heavy metals and arsenic	Blood cells	↑ correlated with copper and cadmium	209
	Toxic acid mining waste rich in heavy metals	Blood cells	↑ (2–10 fold)	210, 212
Turkey	Short term storage	Sperm	↑	213
Green finches	Paraquat	Blood	↑ oxidative damage	214
Broiler chicken	Deoxynivalenol (DON) and mycotoxin	Blood lymphocytes	↑ by DON, ↓ by mycotoxin	215
Turkey and chicken	Aflatoxin B1	Foetal liver cells	↑	216
Chicken	T-2 toxin and deoxynivalenol (DON)	Spleen leukocytes	↑	217
Chicken	Storage conditions (4 °C)	Liver and breast muscle cells	↑ liver cells > breast muscle cells	218
Japanese quails	GSM 900 MHz cellular phone radiation	Embryo cells	↑	219

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Rodents				
Aldh2 knockout mice	Ethanol	Hepatic cells	↑ oxidative damage	220
B6C3F1 mice	Vanadium pentoxide	Lung cells	–	221
C57Bl/6 mice	Straight and tangled multi-walled carbon nanotubes	Lung cells	↑ dose dependent	222
p53$^{+/-}$ mice	Melphalan	Liver, bone marrow, peripheral blood and the distal intestine	DNA crosslinks in all cells tested	223
SKH-1 mice	UV A + Fluoroquinolones (clinafloxacin, lomefloxacin, ciprofloxacin) UVA + 8-methoxypsoralene (8-MOP) Age dynamics	Epidermal cells	↑↑ for fluoroquinolones ↓ for MOP	224
Dyslipidemic *ApoE*$^{-/-}$ mice	Ageing	Aorta, liver and lung	↑ Oxidative damage in liver, – in lung or aorta	225
	Diesel exhaust particles	Aorta, liver and lung	↑ Oxidative damage in liver, – in lung or aorta	226
Balb/c mice	*Trypanosoma cruzi* infection	Peripheral blood, liver, heart and spleen cells	↑ in heart and spleen	227
CD-1 mice	Lead acetate	Nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testes	↑ in all organs on prolonged exposure; – in testes	228
Swiss albino mice	Sanguinarine alkaloid, argemone oil	Blood, bone marrow cells and liver	↑ dose dependent in blood and bone marrow	229, 230
	Cypermethrin	Brain, liver, kidney, bone marrow, blood, spleen, colon	↑	231
	Steviol	Stomach cells, hepatocytes, kidney and testicle cells	↑	232
	Apomorphine	Brain cells	–	233

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Table 1.3 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
	8-oxo-apomorphine-semiquinone	Brain cells	↑	233
	Ethanol, grape seed oligomer and polymer procyanidin fractions	Brain cells	↓ ethanol-induced protection by grape seed	234
	Nonylphenol and/or ionizing radiation	Liver, spleen, femora, lungs and kidneys	↑ in all organ of males, kidney only in females. ↓ with radiation in males, ↑ in female mice	235
Male CBA mice	Pesticide formulations (Bravo and Gesaprim)	Hepatic cells, bone marrow cells spleen cells	↑↑	236
Isogenic mice	Sulfonamide, protozoan parasite *Toxoplasma gondii*	Peripheral blood cells, liver cells and brain cells	↑ in peripheral blood cells	237
Cirrhotic rats	Rutin and quercetin	Bone marrow cells	↑↑	238
Male Sprague–Dawley rats	*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), glycidol, 2,2-bis(bromomethyl)-1,3-propanediol (BMP), 2-nitroanisole (2-NA), benzyl isothiocyanate (BITC), uracil, and melamine	Urinary bladders	↑ with BBN, glycidol and BMP, – with 2-NA, BITC, uracil and melamine	239
In vitro				
FE1 Muta Mouse lung epithelial cell line.	Carbon black	Lung epithelial cell line.	↑	240
Rat Alveolar type II epithelial cells	Cigarette smoke	Lung cells	↑	241
L5178Y mouse lymphoma cells	Ketoprofen, promazine, chlorpromazine, dacarbazine, acridine, lomefloxacin, 8-methoxypsoralen, chlorhexidine, titanium dioxide, octylmethoxycinnamate	Lymphoma cells	Positive with phototoxic compound	242

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Murine primary cultures of brain cells and a continuous cell line of astrocytes	Xanthine and xanthine oxidase, hydrogen peroxide, Superoxide dismutase, catalase, or ascorbic acid.	Brain cells	↓ by antioxidants	243
Chinese hamster ovary (CHO) cell line	Endosulfan	Ovary cells	↑	244
	Cypermethrin, pendimethalin, dichlorovous	Ovary cells	↑	245
Humans clinical				
Breast cancer patients and controls	Radiosensitivity	Peripheral blood mononuclear cells	↑↑ and reduced DNA repair	246, 247
Breast cancer patients and controls	Radiotherapy and/or chemotherapy treatment	Peripheral blood mononuclear cells	↓ post treatment	248
Papillary thyroid cancer (PTC) patients	Basal DNA damage	Peripheral blood lymphocytes	↑	249
Children	Exposed to air pollution	Oral mucosa cells	↑	250
Normal individuals	Chlorhexidine	Buccal epithelial cells and peripheral blood lymphocytes	↑	251
Non-small cell lung cancer (NSCLC) patients	Chemotherapy, Platinum based derivatives for therapy	Lung cells	↑ in patients	252
Ataxia telangiectasia heterozygote	X-irradiation	Peripheral leukocytes	↑ (~3 times higher) in patients	253
Nijmegen breakage syndrome (NBS) patients	X-irradiation	Peripheral blood mononuclear cells	↑ in patients	254
Alzheimer disease patients	–	Peripheral blood mononuclear cells	↑ in patients	255
Breast cancer patients	–	Peripheral blood mononuclear cells	↑ in patients	256
Type 2 diabetes mellitus and healthy males	Oxidative DNA damage	Peripheral blood cells	↑	257
	Exercise training	Peripheral blood cells	↓ in patients	258
Cancer (testicular cancer, lymphoma and leukaemia) patients	DNA integrity	Spermatozoa	Decreased DNA integrity	259

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Table 1.3 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
Dietary intervention				
Healthy subjects	Tomato drink	Blood lymphocytes	↓	260
	Grape juice	Blood lymphocytes	↓	261
	Rosemary and citrus extracts	Blood lymphocytes	↓ damage in UV exposed lymphocytes	262
	Palm date	Faecal water	↓	263
	Green vegetables	Blood lymphocytes	↓	264
Smokers	Vitamin C supplementation	Blood lymphocyte	↓	265
Technical anaesthesiology staff	Vitamin E and vitamin C	Blood lymphocyte	↓ in oxidative damage	266
Colon cancer patients	Flavonoids (Quercetin and rutin)	Blood lymphocyte	↓ in damage induced by PhIP and IQ	267
Occupational				
Airport personnel	Jet fuel vapours, jet fuel combustion products	Exfoliated buccal cells and lymphocytes	↑	268
Agricultural workers	Pesticides	Lymphocytes	–	269
	Pesticides	Lymphocytes	↑	270, 271
Rubber factory workers	Substances used in the rubber industry	Peripheral blood	↓ in exposed subjects	272
	Substances used in the rubber industry	Exfoliated urinary cells	↑	273
Outdoor workers in Mexico cities	Air pollutants	Blood lymphocytes	↑	274
Rickshaw pullers	Exhaustive exercise	Lymphocytes	↑	275
Nuclear medicine personnel	Ionizing radiation	Peripheral blood leukocytes	↑	276
	Ionizing radiation	Peripheral blood leukocytes	↑	277
Print workers	Benzene	Human T- and B-lymphocytes and granulocytes	↑ B-lymphocytes >T-lymphocytes >granulocytes	278

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Workers in battery factory	Lead (Pb) and cadmium (Cd)	Peripheral lymphocytes	↑	279
	Pb	Peripheral lymphocytes	↑	280
Asbestos cement plant workers	Asbestos cement	Peripheral lymphocytes	↑	281
Pesticide factory workers	Fenvalerate exposure	Sperm	↑	282
Footwear workers	Organic solvents	Peripheral blood	↑	283
Coke-oven workers	Coke oven emissions	Blood lymphocytes	↑	284
Welders	Cd, Co, Cr, Ni, and Pb	Lymphocytes	↑	285
Pesticide formulators	Organophosphorus pesticides	Lymphocytes	↑	286
Copper smelters	Inorganic arsenic	Leukocytes	↑	287
Chrome-plating workers	Chromium(VI)	Lymphocytes	↑↑	288
Workers in foundry and pottery	Silica	Lymphocytes	↑	289
Furniture manufacturers	Formaldehyde	Lymphocytes	↑	290
Pharmaceutical industry workers	Phenylhydrazine, ethylene oxide, dichloromethane, and 1,2-dichloroethane	Lymphocytes	↑	291
Farmers	Pesticide, fungicides	B and T lymphocytes	↑	292
Nurses	5-fluorouracil, cytarabine, gemcitabine, cyclophosphamide and ifosfamide	Lymphocytes	Slight ↑	293
Lifestyle				
Normal individuals	Endurance exercise	Lymphocytes	↑	294
Active and passive smokers	Smoking	Lymphocytes	↑	295
Normal individuals	Smoking Diet (vegetarian or non-vegetarian)	Lymphocytes	↑	296–299
Rural Indian women	Biomass fuels	Lymphocytes	↑	300
Normal individuals	Benzo[*a*]pyrene, beta-naphthoflavone (BNF)	Human umbilical vein endothelial cells (HUVEC)	↑	301
In vitro				
Episkin	UV, Lomefloxacin and UV or 4-nitroquinoline-*N*-oxide (4NQO) and protection by Mexoryl	Skin fibroblast cells	↑ reduced by Mexoryl	302
Sperms	Reproductive toxins	Male germ cells	↑	303, 304
Prostate tissues primary culture	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), its *N*-hydroxy metabolite (*N*-OH-PhIP) and benzo[*a*]pyrene (B[*a*]P)	Prostrate cells	↑ dose related	305

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Table 1.3 (*Continued*)

Model	Agent tested	Cell used	DNA damage[a]	Ref.
Human keratinocytes	UVA or UVB	Skin cells	↑	306
MCF-7 cells	Oestradiol	Breast cells	↑ concentration dependent	307
JM1 cells	Oestradiol	Lymphoblast cells	–	307
HepG2 cells	Endosulfan	Liver cells	↑	308
	Indirect acting genotoxins (cyclophosphamide)	Liver cells	↑	309
Mini organ cultures of human inferior nasal turbinate epithelia	Sodium dichromate, *N*-nitrosodiethylamine (NDEA) and *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (MNNG)	Nasal cells	↑ with sodium dichromate and MNNG – with NDEA	310
	Mono(2-ethylhexyl) phthalate (MEHP), benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE), or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG).	Nasal cells	↑ with BPDE and MNNG – with MEHP	311
Human lymphocytes	Heterocyclic amine and prevention by monomeric and dimeric flavanols and black tea polyphenols	Lymphocytes	↓ in oxidative damage	312
	C_{60} Fullerenes	Lymphocytes	↑	313
	Municipal sludge leachates	Lymphocytes	↑	314
	Metabolites in maple syrup urine disease, L-carnitine	Lymphocytes	↑, decreased by L carnitine.	315
	Titanium dioxide (TiO_2) nanoparticles	Lymphocytes	↑	316
HaCaT cells	Citrus and rosemary extracts	Human keratinocytes skin cells	↓ in UV-induced DNA damage	263
HeLa cells	Vitamin C	Epithelial cells	–	317

[a]↑ Significant increase in DNA damage, ↑↑ highly significant increase in DNA damage; ↓ decrease in DNA damage; – no DNA damage reported.

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chemicals in water samples. The effect of tannins[183] and low concentrations (<10 μM) of diaryl tellurides and ebselen—an organoselenium compound[184] in oxidative DNA damage has been studied in nucleated trout (*Oncorhynchus mykiss*) erythrocytes for use of these compounds in biological systems. Kammann *et al.*[181] demonstrated the Comet assay in isolated leukocytes of carp as an *in vitro* model for evaluating genotoxicity of marine sediment extracts and increased sensitivity of the method with use of the DNA repair inhibitor, 1-beta-D-arabinofuranosylcytosine (ara C). The base excision repair Comet assay has been used to examine DNA repair capacity after exposure to coal tar runoff on fish hepatocytes, to examine the clearance of DNA damage caused.[191] The Comet assay with fish cell lines may be a suitable tool for *in vitro* screening of environmental genotoxicity, however, the metabolizing capabilities of the cell line need to be taken into account.

Cryopreservation has been shown to induce DNA strand breaks in spermatozoa of trout,[174,323] gilthead sea bream (*Sparus aurata*[323]) and sea bass (*Dicentrarchus labrax*[324]). The DNA damage was prevented by the addition of cryopreservants such as BSA and dimethyl sulfoxide.[324] These studies have demonstrated the sperm Comet assay to be a useful model for determining the DNA integrity in frozen samples for commercially cultured species. The DNA damage due to xenobiotics as observed in Comet assay is repairable and this DNA repair can also be measured by Comet assay. However, the more permanent alterations caused by genotoxic compounds are not evaluated through the Comet assay. In such cases, amplified fragment length polymorphism (AFLP) has been found to reveal alterations in DNA even after repair was complete, suggesting supplementation of Comet assay with additional methods to get a holistic picture.[325]

These studies have demonstrated the usefulness of the Comet assay in fishes as a model for monitoring genotoxicity of aquatic habitats using these indicator animals.

1.4.1.2 The Comet Assay in Amphibians

The Comet assay in amphibians has been carried out at adult and larval stages for ecogenotoxicity of aquatic environments and studies have been reviewed by de Lapuente *et al.*[9] The animals chosen for the Comet assay, act as sensitive bio-indicators of aquatic and agricultural ecosystems and are either collected from the site (*in situ*) or exposed to chemicals under laboratory or natural conditions.

Erythrocytes from tadpoles of *Rana* species have been used for the assessment of genotoxicity of water bodies as *in situ* sentinel organisms for environmental biomonitoring.[202–204] *R. pipiens* tadpoles collected from industrial sites showed significantly higher ($P < 0.001$) DNA strand breaks than samples of *R. clamitans* tadpoles from agricultural areas while those collected from agricultural regions, showed significantly higher ($P < 0.001$) DNA damage than tadpoles collected from sites of little or no agriculture. The higher levels of DNA damage may be attributed to the pesticides used in the

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agricultural region. Variation in DNA damage due to sampling time[202] and during various metamorphosis states[326] was also observed in the Comet assay. Hence, for biomonitoring environmental genotoxicity using the Comet assay, pooling of early tadpole phases could be helpful. Studies have been conducted on caged tadpoles in areas where the indigenous population is not present, due to ecological imbalance from pollution *e.g.* large lakes and aquatic areas near high industrial activity. *R. clamitans* and the American toad (*Bufo americanus*) tadpoles were caged at the polluted reference site and demonstrated significant ($P < 0.05$) increases in DNA damage.[327] The effects of ionizing radiation,[197] heavy metal pollution[206] and agrochemicals[207] on DNA damage in blood cells of tadpoles as well as adults of toads or frogs have shown that these animals can provide information about the environment that these species inhabit.

Huang *et al.*[196] have shown the genotoxicity of petrochemicals in liver and erythrocytes of toad *Bufo raddeis*. DNA damage was found to be positively correlated to the concentration of petrochemicals in liver, pointing to the fact that liver is the site for metabolism and may be a good marker for studying genotoxicity of compounds which require metabolic activation. The effect of polyploidy on bleomycin-induced DNA damage and repair in *Xenopus laevis* (pseudotetraploid) and *Xenopus tropicalis* (diploid) was studied using the Comet assay.[198] The *X. tropicalis* was more sensitive with a lower capacity for repair than *X. laevis*, showing that polyploidy protects against DNA damage and allows rapid repair, and hence these species may be used as a good model for DNA damage and repair studies.

1.4.1.3 The Comet Assay in Birds

There are few studies involving the Comet assay in birds (Table 1.3). Genetic damage due to a mining accident involving heavy metals has been reported in free-living, nestling white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) from southwestern Spain,[210–212] however, species-specific and intra-species differences were observed. Frankic *et al.*[217] reported that T-2 toxin and deoxynivalenol (DON) induced DNA fragmentation in chicken spleen leukocytes, which was abrogated by dietary nucleotides. The DON induced DNA damage was also shown to reduce with supplementation of Mycofix select[214] in broiler chicken. Sperm cryopreservation is an important genetic resource in the poultry industry for artificial insemination and the Comet assay is helpful in evaluating the DNA integrity of preserved sperms. Kotłowska *et al.*[213] have demonstrated increased DNA fragmentation in turkey sperm after 48 hours of liquid storage, and Gliozzi *et al.*[328] have shown increased DNA fragmentation and decreased motility in chicken spermatozoa after cryopreservation and storage at -196 °C. Faullimel *et al.*[218] showed that the neutral Comet assay could be used to study the impact of freezing and thawing on DNA integrity in breast fillets and liver cells of frozen chicken.

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1.4.1.4 The Comet Assay in Rodents

Mice and rats have been widely used as animal models for the assessment of *in vivo* genotoxicity of chemicals using the Comet assay (Table 1.3). The *in vivo* Comet assay has been recently included in the ICH SR1 guidelines[329] for regulatory genotoxicity testing and is accepted by the UK Committee on Mutagenicity testing of chemicals in food, consumer products and environment[10] as a test for assessing DNA damage. Within a battery of tests, the Comet assay in liver cells can be used as an *in vivo* test along with mammalian bone marrow micronucleus test and AMES test, which has been accepted by international guidelines.[329] A positive result in the *in vivo* Comet assay assumes significance if mutagenic potential of a chemical has already been demonstrated *in vitro*. There are specific guidelines for the performance of the Comet assay *in vivo* for reliable results.[330,331] Recently, the Japanese Center for the Validation of Alternative Methods (JaCVAM), organized an international validation study to evaluate the reliability and relevance of the *in vivo* rat alkaline Comet assay for identifying genotoxic carcinogens, using liver and stomach as target organs. Pre-validation studies were carried out to optimize the test protocol to be used and chemicals to be tested were decided, which would be used in five laboratories for the validation studies.[332,333] The comprehensive data obtained has been published in Mutation Research, Genetic Toxicology and Environmental Mutagenesis (2015, Volumes 786–788, Mutation Research).

Multiple organs of mouse or rat including brain, blood, kidney, lungs, liver and bone marrow have been utilized for the comprehensive understanding of the systemic genotoxicity of chemicals.[231,232,334,335] The most important advantages of the use of Comet assay is that DNA damage in any organ can be evaluated without the need for mitotic activity and that DNA damage in target as well as non-target organs can also be seen.[335] The mouse or rat organs exhibiting increased levels of DNA damage were not necessarily the target organs for carcinogenicity. Therefore, for the prediction of carcinogenicity of a chemical, organ-specific genotoxicity was necessary but not sufficient.[335]

Different routes of exposure in rodents have been used *e.g.* intraperitoneal,[229,231] oral[336,337] and inhalation[221,338] to study the genotoxicity of different chemicals, as the route of exposure is an important determinant of the genotoxicity of a chemical due to its mode of action. The *in vivo* Comet assay helps in hazard identification and assessment of dose–response relationships as well as the mechanistic understanding of a substance's mode of action. Besides being used for testing the genotoxicity of chemicals in laboratory-reared animals, the Comet assay in wild mice can be used as a valuable test in pollution monitoring and environmental conservation.[339]

The *in vivo* Comet assay in rodents is an important test model, for genotoxicity studies, since many rodent carcinogens are also human carcinogens, and hence this model not only provides an insight into the genotoxicity of human carcinogens but is also suited for studying their underlying mechanisms.

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1.4.1.5 The Comet Assay in Humans

The Comet assay is a valuable method for biomonitoring occupational and environmental exposures to genotoxicants in humans and can be used as a tool in risk assessment for hazard characterization[6,8] (Table 1.3). The DNA damage assessed by the Comet assay gives an indication of recent exposure and at an early stage where it could also undergo repair[340] and thus it provides an opportunity for intervention strategies to be implemented in a timely manner. Follow-up studies conducted in the same population after removal of genotoxicant or dietary intervention can detect the extent of reduction in DNA damage.[341] It is a non-invasive technique compared with other techniques (*e.g.* chromosomal aberrations, micronucleus) which require larger samples (~2–3 ml) as well as a proliferating cell population (or cell culture). Human biomonitoring using the Comet assay is advantageous since it is rapid, cost effective, with easy compilation of data and concordance with cytogenetic assays.[6–8]

The assay has been widely used in studying DNA damage and repair in healthy individuals[3,250,342,343] in clinical studies[246–249,344,345] as well as in dietary intervention studies[260–267] and in monitoring the risk of DNA damage resulting from occupational exposures,[268–293,346,347] environmental,[250] oxidative DNA damage[345,348] or lifestyle.[294–301] The wide applications of the assay and factors (*e.g.* age, gender, lifestyle) which can affect the result, have been discussed recently in the ComNet project to establish baseline data on DNA damage for all laboratories.[6] Though white blood cells or lymphocytes are the most frequently used cell type for the Comet assay in human biomonitoring studies,[349] other cells have also been used for the Comet assay *e.g.* epithelial,[350] (including buccal and nasal cells),[2] sperm,[266–268,282,351,352] urothelial cells[273] and placental cells.[353]

The Comet assay has been used as a test to predict the risk for development of diseases (renal cell carcinoma, cancers of the bladder, oesophagus and lung) due to susceptibility of the individual to DNA damage.[354–356] The *in vitro* Comet assay is proposed as an alternative to cytogenetic assays in early genotoxicity or photogenotoxicity screening of drug candidates[357,358] as well for neurotoxicity. Certain factors like age, diet, lifestyle (alcohol and smoking) as well as diseases have been shown to influence the Comet assay parameters and for interpretation of responses, these factors need to be accounted for during monitoring of human genotoxicity.[3,8]

Human biomonitoring studies using the Comet assay provide an efficient tool for measuring human exposure to genotoxicants, thus helping in risk assessment and hazard identification.

1.5 The Specificity, Sensitivity and Limitations of the Comet Assay

The Comet assay has found worldwide acceptance for detecting DNA damage and repair in prokaryotic and eukaryotic cells.[359] However, issues relating to

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the specificity, sensitivity and limitations of the assay need to be addressed before it gets accepted in the regulatory framework, including inter-laboratory validation of *in vitro* and *in vivo* Comet assay. Though the *in vivo* assay has recently been implemented in regulatory toxicity testing, the *in vitro* assay is not included.[360]

The variability in the results of the Comet assay is largely due to its sensitivity and minor differences in the experimental conditions used by various laboratories as well as the effect of confounding factors in human studies (lifestyle, age, diet, inter-individual and seasonal variation). Cell to cell,[361] gel to gel, culture to culture and animal to animal variability as well as use of various image analysis systems or visual scoring,[362] number of cells scored[363] and use of different Comet parameters,[364] *e.g.* Olive tail moment and tail (%) DNA, are the other factors contributing to inter-laboratory differences in the results, which can be controlled.[365,366] A multi-laboratory DNA base-excision repair study, in three cell lines using the modified Comet assay also showed large inter-laboratory variation attributed to the cell extract and substrate cells incubation step.[367]

The limitation of the Comet assay is that it only detects DNA damage in the form of strand breaks. The alkaline (pH >13) version of the assay assesses direct DNA damage or alkali-labile sites; base oxidation and DNA adduct formation can measured with the use of lesion-specific enzymes.[3] These enzymes are bacterial glycosylase or endonuclease enzymes, which recognize a particular type of damage and convert it into a break that can then be measured in the Comet assay. Hence, broad classes of oxidative DNA damage, alkylations and ultraviolet-light-induced photoproducts can be detected as increased amounts of DNA in the tail. Oxidized pyrimidines are detected with use of endonuclease III, while oxidized purines are detected with formamidopyrimidine DNA glycosylase (FPG). Modifications have been made in the protocol[3,331] to specifically detect double-strand breaks (neutral Comet assay), single-strand breaks (at pH 12.1), DNA crosslinking (decrease in DNA migration due to crosslinks) and apoptosis. The neutral Comet assay also helps to distinguish apoptosis from necrosis, as evidenced by the increased Comet score in apoptotic cells and the almost zero Comet score in necrotic cells.[368] An adaptation of the Comet assay was also developed which enables the discrimination of viable, apoptotic and necrotic single cells.[369] DNA repair can also be measured using the Comet assay and has been reviewed.[370] With integration of biological and engineering principles, a Comet chip has been devised, which potentiates robust and sensitive measurements of DNA damage in human cells and can be utilized for various applications of the Comet assay.[371] The Comet–FISH assay was successful in detecting damage and repair in different genes regions in a cell and could be used for clinical purposes.[372]

Tail (%) DNA and Olive tail moment (OTM) give a good correlation in genotoxicity studies[41] and since most studies have reported these Comet parameters, it has been recommended that both these parameters should be

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applied for routine use. Since the OTM is reported as arbitrary units and different image analysis systems give different values, tail (%) DNA is a considered a better parameter.[364]

It is therefore required that the *in vitro* and *in vivo* testing be conducted according to the Comet assay guidelines and that appropriately designed multi-laboratory international validation studies should be carried out. Guidelines for the *in vitro* as well as *in vivo* Comet assay have been formulated.[373,374] Study design and data analysis in the Comet assay have been discussed by the International Workgroup on Genotoxicity Testing (IWGT), where recommendations were made for a standardized protocol, which would be acceptable to international agencies.[375] Critical parameters of the protocol, sensitivity of the protocol used, combination and integration with other *in vivo* studies, use of different tissues, freezing of samples and choice of appropriate measures of cytotoxicity were some of the areas covered in the recommendations.[375]

The *in vivo* Comet assay was the first-tier screening assay for assessment of DNA damage in rodents by the Committee on Mutagenicity, UK.[10] International validation studies with genotoxic chemicals were carried out by the Japanese Centre for Validation of Alternative Methods (JaCVAM),[332,376] supported by the U.S. NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Environmental Mutagen Society/Mammalian Mutagenesis Study Group (JEMS/MMS).

Multi-laboratory validation studies in the European countries have been conducted to study the FPG-sensitive sites and background level of base oxidation in DNA using the Comet assay, in human lymphocytes.[367,377] It was found that half of the laboratories demonstrated a dose–response effect.[377] However, many laboratories have carried out their own validation studies of DNA damage to optimize their research work. The large number of biomonitoring studies have indicated that the Comet assay is a useful tool for detecting exposure and its validation status as a biomarker in biomonitoring is dependent on its performance in cohort studies.

1.6 Conclusions

The Comet assay is now well established and its versatility has imparted a sensitive tool to toxicologists for assessing DNA damage and repair. This has been demonstrated by its wide applications in assessing genotoxicity in plant and animal models, both aquatic and terrestrial, in a variety of organisms, tissues and cell types. *In vitro*, *in vivo, in situ* and biomonitoring studies using the Comet assay have proved it to be a "*Rossetta Stone*" in Genetic Toxicology.

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