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Comet Assay on Toxicogenetics; Several Studies in Recent Years on Several Genotoxicological Agents

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Abstract

Cancer is one of the main causes of death in the world. Prolonged exposure to genotoxic chemicals observed is one of the primary causes of cancer. A number of assays exist for detection of genotoxicity in a variety of experimental systems. The Comet assay also known as the single cell gel electrophoresis assay is used to detect DNA damage as an indicator of exposure to geno toxicogical agents. The Comet assay is a broadly used method in human, environmental, and eco genotoxicological studies. The aim of our review is to describe Comet Assay protocol with the advantages and limits and to develop some recent studies in which the authors have used comet test to confirm or reverse agent's genotoxicity.

Keywords: Comet test; Toxicogenetics; DNA damage; Genotoxicological agents

Introduction

Cancer is one of the main causes of death in the world, and a major issue for human health. Prolonged exposure to a number of chemicals was observed to be one of the primary causes of cancer [1]. The monitoring of the surrounding environment for chemicals and compounds with possible genotoxic activity is of high priority [2]. Thus, the development of instruments for identifying risky chemicals and the understanding of their toxicity mecanism is a major objective for scientific research [3]. A number of assays exist for detection of genotoxicity in a variety of experimental systems, some of them with limited use due to complicated technical setup, the single cell gel electrophoresis assay also defines the Comet assay [3], discovered for the first time in 1984 by two Swedish researchers, Ostling and Johanson [4]. In 1988 Singh et al., introduced the concept of alkaline version [2,4]. It allows investigation of DNA damage in virtually all cell types without the necessity of cell cultures [2]. It is widely used to detect DNA damage [5] as an indicator of exposure to genotoxicogical agents [2,6,7]. The Comet assay is a used method in human, environmental, and ecogenotoxicological studies [2] and it is performed to detect genotoxicity effect of biocides, chemicals prodcuts, agrochemicals, pharmaceuticals and food additives in genotoxicity assaying [8].

Principle and Methodology of Comet Assay

The comet assay is widely used to assess DNA damage. Before lysis and leaving nucleoids, cell should embedding in agarose than fixed on microscope slide. After an alkalin electrophoresis, DNA loops containing breaks are relaxed and extend towards the anode, forming a comet-like image viewed by fluorescence microscopy with a suitable stain [9,10]. The different steps of Comet assay are Preparation of microscope slides: The aim of microscope slide preparation is to ensure the uniformity of the gel, assure the stability and the survival for the collection of data, minimise background noise as well as to ensure well visualized of comets [11].

Release of DNA from lysed cells

Apply on the slides, a lysis solution that contain Triton X-100 and a high concentration of salt with 2.5 M NaCl [4,12]. Lysis allaow the removing membranes, releases the soluble components of the cell, strips histones from DNA, and sheets of compact structures that are nucleoids wherein the DNA is attached at intervals to the nuclear matrix [13].

Exposure to alkalin and electrophoresis (pH 13)

The supercoiled DNA is attached to a nuclear matrix creating a structure called a "nucleoid." [4,12]. After treatment with lysis buffer, slides were incubated for 20 min in a freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA; pH>13) [12], before DNA was submitted to electrophoresis (20 min; 25 V; 0.90 V/cm; 300 mA) [14]. Under electrophoresis, DNA is attracted to the anode, only the loops containing breaks, relaxing supercoiling, able to significantly expand [13].

Comet visualization of DNA staining

This step depend on investigator-specific needs and probably have little effect on reliability and sensitivity. The fluorescent dyes often used are propidium iodide, ethidium bromide, 4,6- diamidino-2-phenylindole (DAPI), and YOYO-1 (benzoxazolium-4-quinolinum oxazole yellow homodimer) and SYBR Green [4,11].

Comet scoring

The collecting of comet data is based on the application of image analysis techniques to individual cells, other programs are also usable and available. Many researcher have automated some methods and techniques and developed an automated analysis of comet data. The determination of the proportion of cells in which theirs migrations on gel are alterated is the simplest and the useful method [11].

In the study of Azqueta et al., they found that the following conditions should give acceptable results: 0.6–0.8% (final) agarose concentration; 40 min alkaline incubation before electrophoresis for 20 min at about 1.15V/cm across the platform, or for 30 min at about 0.83V/cm. [5]. The table below summarize different step of comet assay (Figure 1).

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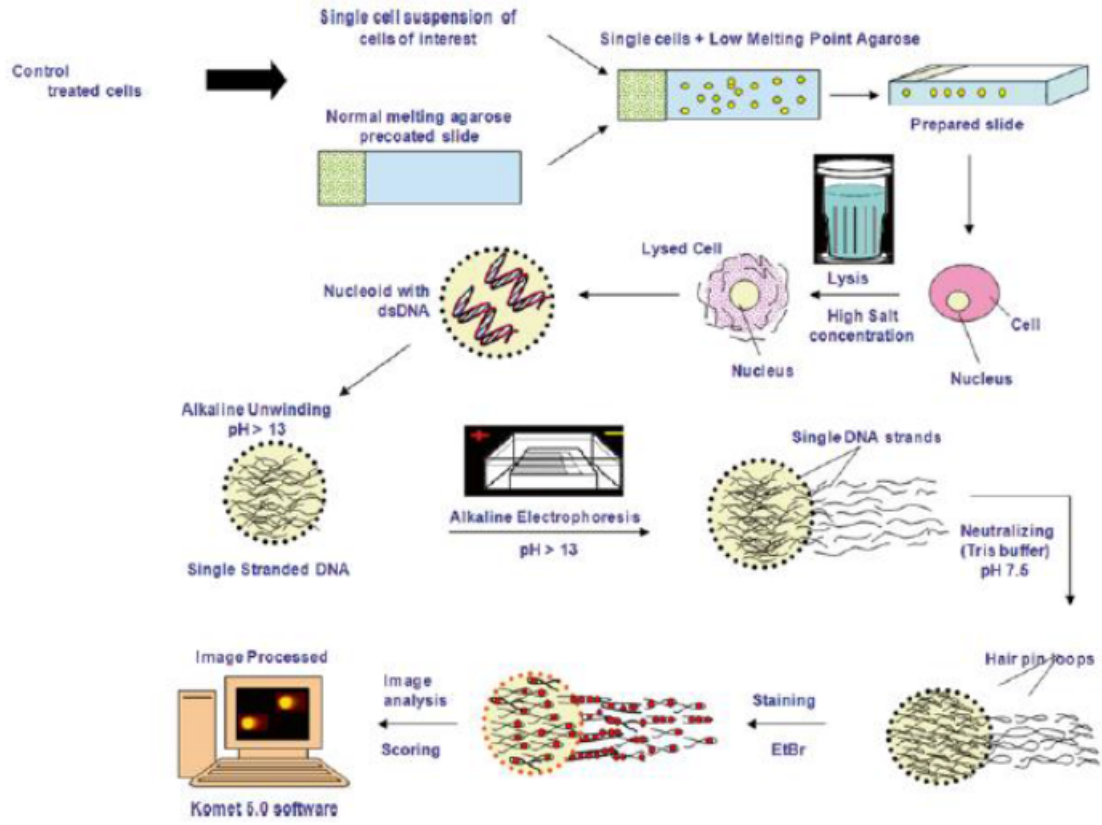

Figure 1: Schematic showing the process of comet assay [16].

Advanages and Limits of Comet Assay

Comet assay is currently also frequently used in human biomonitoring to detect genotoxic effects in human populations exposed to genotoxins in the environment or at the workplace [8]. The Comet assay is a broadly used method in environmental and ecogenotoxicological studies [2]. This technique is very sensitive, to measure DNA damage in early stages at the level of the individual cell [12,13]. Comet Assay is technically simple, relatively, fast, cheap [2,5,15-20] and requiring only a small number of cells [21]. Comet assay acquired as the standard method and technique to assess damage or repair of DNA [9], genotoxicity testing and biomonitoring. As a test of genotoxicity Comet assay can be used to identify carcinogens and human mutagens [19]. In its alkaline version the assay enables sensitive detection of a several variety of DNA damage such as single and doublestrand DNA breaks, DNA– protein crosslinks, sites undergoing DNA repair and/or apoptos [2,4].

Major limit of comet assay is the restriction of the samples number that can be processed in an experience, it is due to the size of the platform electrophoresis [20,22]. Comet Assay cannot directly distinguish between the dead cells and the cells that have a heavily damaged DNA [23].

Recent modifications of the assay have increased throughput, essentially by reducing the gel size. Rather than creating large gels with containing a few hundred cells and thousands of cells, have been applied to glass slides [4].

In a recent study, an automated platform with high content imaging endpoints for cell viability, oxidative stress and DNA damage, in combination with the high throughput comet assay, was successfully employed to measure DNA damage caused by coated and un-coated iron oxide NPs in two mammalian fibroblast lines showing that these methods provide a fast way to determine NP toxicity [24]. In another study, a high-throughput Comet Chip screening assay based on a microfabricated 96-well design with automated processing was used to evaluate DNA damage caused by ZnO, Ag, Fe_2O_3, CeO_2, and SiO_2 NPs in human lymphoblastoid (TK6) cells in suspension, and adherent Chinese hamster ovary (H9T3) cells [25]. High throughput comet assay approaches, whether 12 mini-gels on a slide, 48 or 96 mini-gels on a GelBond film [6,7,24], or microfabricated 96-well CometChips [25], all show robustness, improved efficiency and reduced processing time. In addition, they reduce the risk of user bias in comparison with the standard comet assay (Table 1 and Figure 2).

Recents Studies Using Comest Assay

In recent years, various studies used comet assay was developed, researchers have used this test to evaluate the genotoxic effects of many agents. A clinical study in 60 CKD patients led by Mamur, Unal et al., to investigate the possible role of Diabetes mellitus (DM) in CKD patients with respect to DNA damage. DNA damage measured by the comet assay was significantly higher in CKD patients than in controls group. No difference in comet tail length or tail intensity was found between diabetic and non-diabetic individuals. Neither comet tail length nor intensity was observed with clinical and paraclinical studied parameters (Age, sex, hemoglobin, hypertension, duration of hemodialysis, and ferritin levels) [26]. In another study of Ramy, Ghany et al., impact of hyperbilirubinemia and two different types of phototherapy were studied to assess DNA damage using Comet assay on blood mononuclear

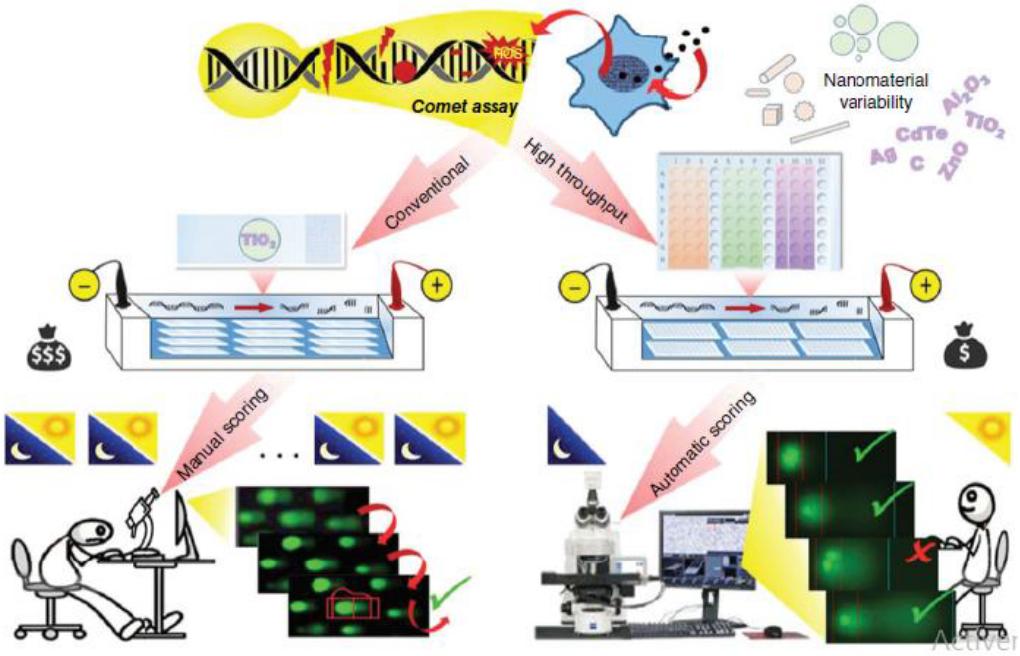

Figure 2: Comparison between HTS and conventional comet assay, savings in time and cost are remarkable [13].

Step	Duration	Temperature	Solution	Remarks
Dilution in LMPA		≈ 37°C	0.5-1% LMPA in PBS or KBSS	Dilute cell suspension in molten LMPA (1:100).
Preparation of slides		Room (≈ 20°C)		Place 2 × 75-80 µL of cells in LMPA pre-coated slide.
Place coverslip – Solidification– Remove coverslip	15 min	4°C		
Lysis	30 min to 1h	4°C	0.45 M NaCl (m/v); 40 mM EDTA (m/v); 5 mM Tris (pH 10).	Add 10% v/v dimethylsulfoxide and 1% v/v Triton X-100 just before use. Do not re-use buffer.
DNA unwinding	40 min	4°C	0.1 µM EDTA; 0.3M NaOH (pH ≈13)	Run electrophoresis at 25 V. Do not use buffer more than twice.
Electrophoresis	30 min	4°C	0.1 µM EDTA; 0.3M NaOH (pH ≈13)	
Neutralising	15 min	4°C	0.2 M Tris-HCl buffer (pH 7.5)	
Fixation	10 min	4°C	Absolute methanol Ethidium bromide 0.02	Optional. The staining time allows the dye to bind to DNA,
Stain	5-10 min	Room (≈20°C)	mg/L in water	otherwise bleaching of the dye exposed to UV will be almost immediate. If working with pre-fixed, dry, slides, rehydrate in cold water for at least 15 min. Do not wash the slides after staining. Mount with coverslip.

Table 1: Standard comet assay protocol [15].

cells of neonates. In their work on term neonates with non-hemolytic hyperbilirubinemia and control healthy neonates were included and blood collected in all infants and after intensive or conventional phototherapy in jaundiced infants. There is non-significant difference between jaundiced and non-jaundiced neonates on DNA damage. It increased significantly after exposure to phototherapy compared with prephototherapy values. The duration of phototherapy correlated positively with markers of DNA damage but the intensity of used light don't have any genotoxic impact [27] (Figure 4).

In ecotoxicological studies, Zapata et al., Evaluated genotoxic biomarkers of many pollutants; industrial, agricultural, and mining activities in Colombia, they used erythrocytes of *Trachemys. callirostris* (turtle species endemic) in order to know the levels of DNA damage in this species and demonstrated its possible use for screening genotoxic effects in freshwater environments. Results showed that the frequency of micronuclei in the samples was positively related to comet tail length and tail moment and that both genotoxicity biomarkers may be applied to *T. callirostris* erythrocytes as a species of choice for the evaluation of environmental pollutants effects in freshwater [28]. Mellado-Garcia, Puerto et al., used Comet assay in liver and stomach, and Micronucleus Test in bone marrow to assess the genotoxicity of propyl thiosulphinate oxide (PTSO); principal constituent of Allium

species, in rats by oral administration (doses: 5.5, 17.4, and 55 mg/kg) to let it used in the food packaging industry. They confirmed that PTSO don't have any genotoxicity effects and they only found slight modifications in histopathological analysis highest dose [29]. In the study of Cetinkaya, Ercin et al., utility of comet assay was demonsrated in foods experiments, they measured quarantine doses in an irradiated citrus fruits exposed to 0.1-1.5 kGy. To interpretate comet assay tail length, tail moment and tail DNA% of comets were used. Irradiated citrus fruits showed the separated tails from the head of the comet by increasing applied doses from 0.1 to 1.5 kGy [30]. Based on comet assay, micronucleus test and SHE transformation assay, Darne, Coulais et al., compare the genotoxic and the carcinogenic potential of crystalline and amorphous silica particles in Syrian Hamster Embryo SHE cells. Particles studies were; natural amorphous silica, partially crystallized silica and quartz silica. In addition, silica samples were also tested with the same genotoxicity assays in V79 hamster-lung cells. In the micronucleus and the comet assays, none of the silica was capable to cause genotoxic effects in SHE cells and only the amorphous silica was found positive in V79 cells. In the SHE cell transformation assays, the partially crystallized and quartz silica were able to induce morphological cell transformation [31]. Nafee, Saeed et al., assessed the effect of very low dose of fast neutrons on the chromatin and DNA of rats' peripheral blood mononuclear cells (PBMC) and leukocytes by Fourier transform infrared (FTIR) and comet assay. They used and irradiated group with neutrons of 0.9 cGy against no irradited group. The analysis of FTIR spectra of the PBMC showed increased in the area of phosphodiesters of nucleic acids and the area ratios of RNA/DNA and phosphodiesters/carbohydrates, significant increase in the areas of RNA, significant decrease in the areas of DNA ribose and different tail lengths presented in leukocytes [32]. Mukherjee et al., investigated people of southern Assam consuming arsenic contaminated water and chewing tobacco to determine the genotoxic effects of arsenic employing the buccal cytome assay. The results obtained in the study showed that arsenic significantly induced nuclear damage, increases micronucleus and other cytome parameter in buccal cells induced by tobacco [33]. Balikci et al., assessed the genotoxic and the antigenotoxic effects of methanol extract of folk medicine plant (*Hypericum adenotrichum* Spach) in human lymphocyte culture using comet assays, *in vitro* sister chromatid exchange and micronucleus test. Their result showed that at (125-500 µg/mL) doses, the *H. adenotrichum* extract induce significant genotoxic activity in human lymphocytes, and anti-growth effects on cancer cell lines between 0.2 and 100 µg/mL concentrations. The mode of cell death in cancer cells was shown to be apoptosis due to the presence of pyknotic nuclei [34]. In the study of da Silva et al., DNA damage was analyzed by the Comet assay and by the micronucleus (MN) test. The damage frequency, damage index, the MN frequency, and the activity of superoxide dismutase (SOD) activity were elevated in pesticides exposed agricultural workers than the unexposed group [14]. Another study on genotoxic effect of pesticides led by Carbajal-Lopez, Gomez-Arroyo et al., in Guerrero, Mexico using the comet assay and the micronucleus test in exfoliated buccal cells demonstrated that the tail migration of DNA and the frequency of MN increased significantly in the exposed group, which also nuclear anomalies associated [35]. Employing human hepatoma cell lines (HepG2) Cabral-de-Mello et al., assessed the genotoxic effect of two pesticides (the imidacloprid, sulfentrazone) alone and mixed, they employee comet assay, micronucleus (MN) test, fluorescence in situ hybridization (FISH) abd Salmonella/microsome assay as a mutagenicity test. For all concentrations, significant inductions of MN in HepG2 cells by both pesticides was observed. Imidacloprid alone or mixed with sulfentrazone presented significant response in the comet assay, but not by the MN test. According to the FISH results, the damage caused by imidacloprid and sulfentrazone resulted from a clastogenic action. No significant result was found with Ames test [36] (Figure 3).

In the recent years different studies have evaluated the genotoxicity of many nanomaterials. Comet assay is one of the laborious approaches for evaluating their genotoxicity [25]. Schulz et al., studied *in vitro* and *in vivo* genotoxic effects of differentl sized SiO_2 nanoparticles with alkaline Comet assay and Alkaline unwinding assay. Employing rat

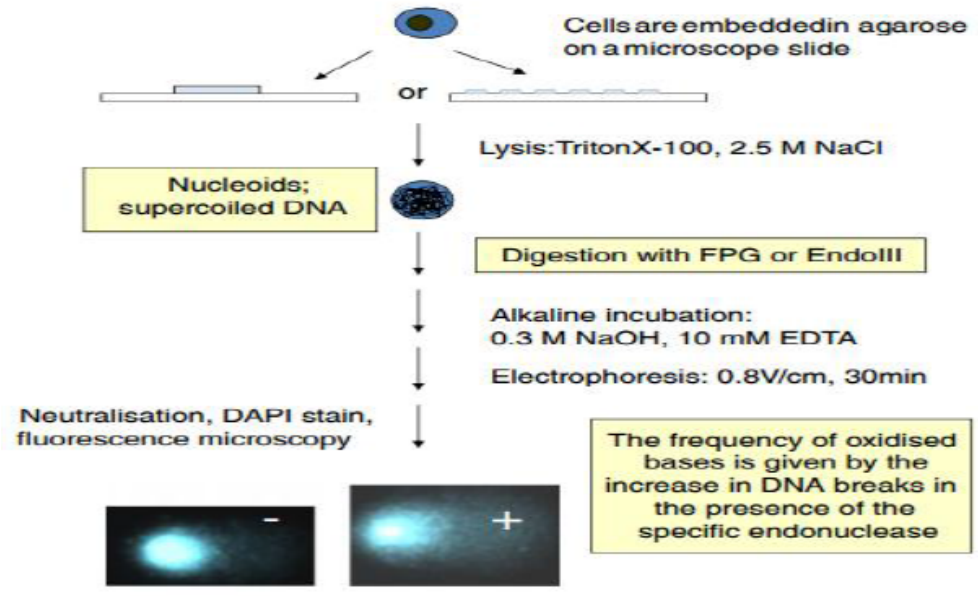

Figure 3: Schematic representation of the comet assay with enzyme modification for the detection of DNA damage [18].

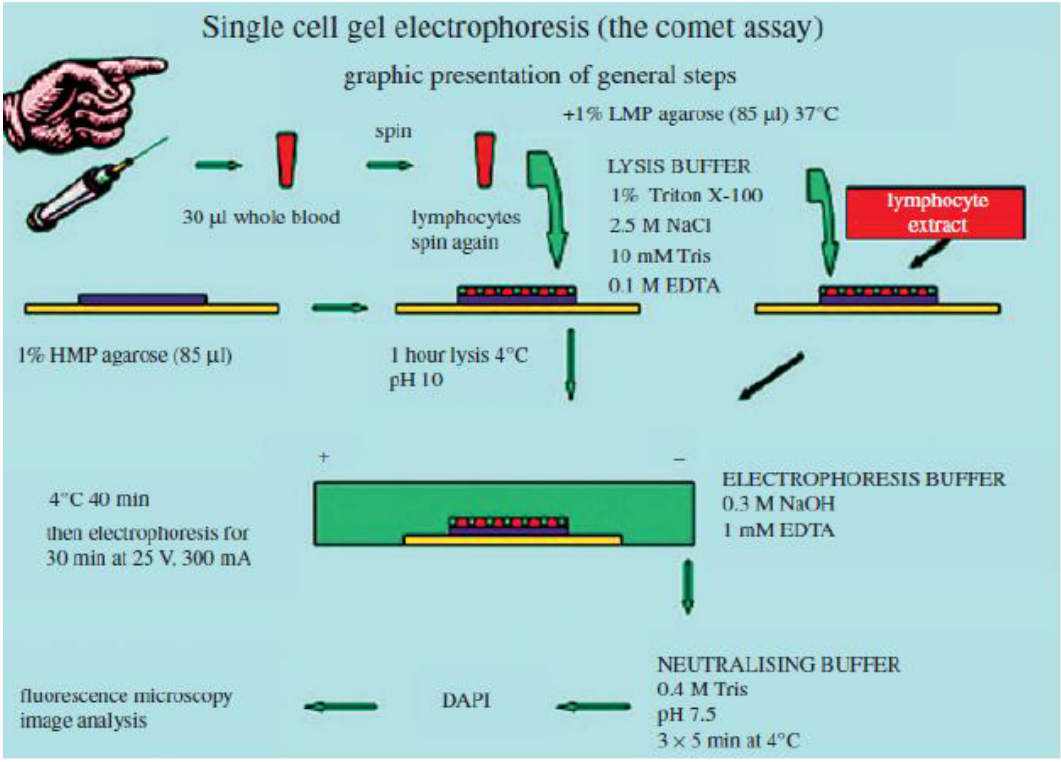

Figure 4: Graphic presentation of general steps of comet assay in clinical studies [17].

precision cut lung slices, the Comet assay showed that 15 nm-SiO_2 and 55 nm-SiO_2 induced s DNA damage at ≥100 µg/mL. Using A549 cells, In the Alkaline unwinding assay, increases in DNA strand breaks was observed with 30 nm-SiO_2 and 55 nm- SiO_2 at ≥50 µg/mL. No genotoxic effects observed *in vivo* Employing rat lung or in the bone marrow with 15 nm-SiO_2 and 55 nm-SiO_2 [37]. Another study was led by Battal, Celik et al.. to evaluate SiO_2 (particle size 6, 20 , 50 nm) nanoparticules genotoxicity. Comet assay, sister chromatid exchange, and cytokinesis block micronucleus test, were employed *in vitro* in cultured blood lymphocytes. It is found that SiO_2 nanoparticles at different size (6, 20, 50 nm) decreased Mitotic index (MI), Cytokinesis block proliferation index (CBPI), increased the sister chromatid exchange frequency (SCE) and caused DNA damage [38]. Jain et al., evaluated apoptotic and genotoxic effect of $Cr_2 O_3$ NPs in human lung epithelial cells (A549) by comet assay and cytokinesis block micronucleus assay. They deduced that $Cr_2 O_3$ NPs led to the increasing levels of reactive oxygen species. Indeed the oxygen species led to DNA damage, decreasing in mitochondrial membrane potential and increase the ratio of BAX/Bcl-2 which ultimately cause cells death [39]. In the study of Akca et al., Comet assay, with and without the use of FPG enzyme, micronucleus assay and the soft-agar colony assay were used to evaluate oftitanium dioxide nanoparticles genotoxic effect. Human embryonic kidney and mouse embryonic fibroblast cell were used. Genotoxic effects were only found with higher dose (1000 µg/mL) [40].

Wang et al., measured cytotoxicity, DNA damage, and apoptosis can be induced by TiO_2 NPs (5 nm) in A549 cells. In exposed cells 50 to 200 µg/mL TiO_2 concentration caused DNA damage (the comet assay), an apoptotic morphological feature and apoptotic bodies, a significant induction in micronucleus formation. With TiO_2 NPs of 100 and 200 µg/mL concentrations for 48 h a significant G2/M phase arrest and an increased proportion of apoptotic cells was found by Flow cytometric analysis. In the same concentrations quantitative real-time PCR (qRT-PCR) indicated that the expression of caspase-3 and caspase-9 messenger RNA (mRNA) was increased significantly [41]. Khoei et al., used alkaline comet assay, in a spheroid culture model of HT-29 colon cancer cell lines, to assess the effect PLGA nanoparticles as a 5-fluorouracil (5-FU) carrier. Results showed that nanoparticles as 5-FU carriers importantly caused the most DNA damage, combined with the iron oxide core PLGA nanoparticles induced more DNA damage than without the iron oxide core [42]. In the work of Zijno, De Angelis et al., the genotoxicity of zinc oxide and titanium dioxide nanoparticles (ZnO NPs; TiO_2 NPs) was measured by micronucleus and Comet assays in human colon carcinoma cells (Caco-2 cells. They found that ZnO NPs induced micronucleus and DNA damage and that Caco-2 cells incubated with ZnO NPs were not able to repair the oxidative DNA damage and that was efficiently repaired after treatment with TiO_2 NPs [43]. In the study of Rinna, Magdolenova et al., Comet assay is used for to detect the human 8-oxoguanine DNA N-glycosylase 1 hOGG1 and demonstrate the genotoxicity of gold naoparticles. They observed that AgNP induced DNA oxidation via a mechanism involving ROS formation after 30-min treatment, whereas the response was negative after 2h [44]. Woodruff et al., assessed the genotoxicity of 10 nm Titanium dioxide nanoparticles (TiO_2-NPs) using Ames test and Comet assay. No mutation induction was found with the Ames test. For the Comet assay, TK6 cells were treated with 0-200 µg/mL TiO_2-NPs for 24 h at 37°C. Although the TK6 cells did take up TiO_2-NPs, and no significant induction of DNA breakage or oxidative DNA damage was observed in the treated cells [45]. Genotoxicity of Titanium dioxide nanoparticles (TiO(2) nanopartilcles was also evaluated in another study of Shukla, Sharma et al., Using human epidermal cells (A431). Indeed they demonsrated that TiO(2) NPs induced ROS and oxidative stress leading to oxidative DNA damage and the formation

of micronucleus, a probable mechanism of genotoxicity [46]. In the study of Ponnuraj et al., the cytotoxicity and genotoxicity of dental nanocomposite (KelFil) was reversed with MTT assay, comet assay and chromosome aberration tests using the human lung fibroblast cell line (MRC-5). In the comet assay, no comet formation was found in the KelFil groups. Similarly, no significant aberrations in chromosomes were noticed in KelFil groups [47].

In food industry Mellado-Garcia, Maisanaba et al., used many genotoxic systems (the comet assay, the micronucleus test, the mouse lymphoma thymidine-kinase assay and Ames test) to evaluate mutagenicity and genotoxicity potential of propyl thiosulfinate oxide (PTSO) (0-50 μM). No micronucleus was found, the comet assay indicated that PTSO did not induce DNA breaks or damage and was not mutagenic in the Ames test, however, it was mutagenic in the MLA assay after 24 h of treatment (2.5-20 μM) [48].

Comet assay was widely applied in molecular toxicology, epidemiological studies and other domain [49]. Hermeto et al., have evaluated pH effects on genomic integrity in adipose-derived mesenchymal stem cells (MSCs); with the aim of evaluating two different comet assay protocols for genomic damage pattern analysis in MSCs derived from adipose tissue [50]. They found that in the pH>13 alkaline version detects damage in the form of single-strand breaks, double alkali-labile sites, cross-linking, and loss of excision. The other version, pH=12 detects mainly single- and double-strand breaks [50].

For a more physiological approach, by the use of cumulus-oocyte complexes (COC-CA), Greco et al., have used comet test to identify the genotoxicity of environmental agents on oocytes [51]. Removal from oviducts and after the ovulation they exposed COC directly exogenous factors. In their study they used three control in which one is negative and two other positive; the first positive control exposed to the COC is hydrogen peroxide (H_2O_2), and they incubated cerium dioxide nanoparticles (CeO_2 NPs) with the other group. By comparison with negative control, the DNA damage is observed with COC incubated respectively, with H_2O_2 and CeO_2 NPS [51] Venancio et al., determined the genotoxicity of the Quinoline yellow used in cosmetic industry employing comet assay and cytokinesis-block micronucleus cytome assay in HepG2 cell line, which closely. The results demonstrated that low (from 0.5 to 20 μg/mL) QY concentrations were genotoxic in HepG2 cells on comet and micronucleus assays [52].

To analysis the oxidation of DNA in cell lines, Zhao et al., used a modified comet assay; lesion-specific endonucleases-modified comet assay (FPG-comet assay), they demonstrated in their study the sensibility and the efficiency of the FPG comet assay for measuring oxidative DNA damage caused by the exposure to gentoxic agents [49]. Attia et al., led a study through an *in vivo* comet assay and MN test at the recommended human doses to evaluate *in vivo* the genetic damage of citalopram in mice somatic cell [19]. A higher level of DNA-stand breaking was observed with a citalopram multiple doses of 6,12 and 24 mg kg^{-1} day^{-1}, in comparison to those of the corresponding solvent control group and the two increased doses of citalopram at both sampling times gave the more DNA strand breaking, the response was higher at 3 h after treatment than at 24 h, suggesting that there was a time-dependent reduction in DNA damage, which may also suggest a time-dependent DNA damage repair [19].

New method based on comet assay with fluorescence in situ hybridization (FISH) invented by Hanawalt et al., to measure the level of DNA lesions in the genome. A single-stranded probes targeting the termini of DNA segments was synthesized of interest using a polymerase chain reaction-based method. These probes allow the detection of many damage at the single-molecule level, as the lesions are converted to DNA strand breaks by lesion-specific endonucleases or glycosylases [53].

The comparison between two recent developments comet assay, namely 12 minigels per slide, and a format with 96 minigels on GelBond_ film in the study of Azqueta, Gutzkow et al., demonstrated that the level of DNA damage was not affected by the format and give very similar results used in X-rays or methylmethanesulphonate (MMS) treated cells [20]. Feng et al., developed a novel agarose-based microfluidic comets assay method with more rapidity, simplicity, highly reproducibility and of ultra-high throughput, providing a highly efficient approach of choice for single cell genomics than the traditional comet assay [54].

Munari et al., Led an *in vitro* and *in vivo* study to evaluate the genotoxic and antigenotoxic potential of ursnic acide (UA). In this work they used comet assay and micronucleus test with V79 cell cultures incubated with 15, 30, 60, and 120μg/mL UA and Swiss mice treated with 25, 50, 100, and 200 mg/kg UA body weight. Using V79 cells they observed that UA caused DNA damage with doses of 60 and 120 μg/mL and no gentoxic effect with the micronucleus test. *In vivo* result doesn't show any genotoxic effect. They used the same concentrations of UA with methyl methanesulfonate to assess antigenotoxicity and they found that UA mixed with MMS reduced the frequencies of micronuclei and DNA damage *in vitro* and *in vivo* when compared to MMS alone [55]. Oliveira et al., employee comet and micronucleus assay to determine genotoxicity and mutagenicity of Phytoestrogen using mouse peripheral blood. Phytoestrogen (0.083, 0.83 and 8.3 mg/kg body weight) with and without cyclophosphamide was evaluated. They observed that Phytoestrogen reduced DNA damage, reduced cyclophosphamide-induced DNA damage and was not mutagenic. Phytoestrogen was not mutagenic and reduced cyclophosphamide-induced DNA damage. The results from [56]. Switalla et al., studied a new model of comet assay for evaluation of genotoxicity effect of many suspect agents; for their study, they used Precision-cut lung slices (PCLSs) to supply three-dimensional culture. They exposed mirine PCLSto higher doses of ethyl methane sulfonate (0.03–0.4%) and formalin (0.5–5 mM). After exposure to EMS, they found that DNA single strand breaks were increased dose dependently and significantly. In contrast a significant DNA cross links was induced by the exposure to the formalin [57]. Charles et al., proposed to investigate possible uses of the comet assay for the screening of natural products modulating DNA repair, taking different classes of flavonoids as model compounds. At 24 h, the flavone apigenin was found to reduce DNA fragmentation. The flavonol quercetin substantially increases DNA fragmentation at 12 h which may correspond either to direct DNA damage or to the induction of non-specific endonuclease activity [58].

Park et al., used human cells to identify the alteration of DNA and evaluate its damage levels, B-and T-lymphocytes got by magnetic cell sorting MACS were incubated with methyl methane sulfonate (MMS; 5, 25 and 50 μM) and hydrogen peroxide H_2O_2; 5, 25 and 50 μM) [59]. In both B and T cells DNA damage levels were found significant and dose-dependent, importantly DNA damage level in B lymphocytes was higher than in T lymphocytes [59]. Many chemicals compounds were used by Toyoizumi et al., to assess the specificity and the sensibility of skin comet assay, chemicals treated used were N-methyl-N -nitro-N-nitrosoguanidine (MNNG, 0.0125–0.2%), 4-nitroquinoline-1-oxide (4NQO,0.01–0.25%), mitomycinC(MMC,0.0125–0.05%), benzo[a] pyrene (B[a]P, 0.25–2%), and 7,12-dimethylbenz[a]anthracene

(DMBA, 0.25–1%) and they applied it to the dorsal skin of hairless male mice [23]. In their study they confirmed that *in vivo* skin comet assay utile technique able to detect DNA damage induced on the skin by chemicals genotoxic [60]. In the study of Berthelot-Ricou et al., they used three groups of 40 mouse oocyte to evaluate the genotoxicity of 1,2-propanediol (PrOH). Mouse oocytes were treated with different concentration of PrOH (5%, 7.5%, and 15%) in which every concentration was tested during short and long time (1-2 h and 1-5 min) in comparison with control group. They found that the alteration of DNA depends only on the high concentration of PrOH (7.5% and 15%) whatever the treatment duration [61]. Benkovic et al., evaluated the genotoxicity of duplicated doses of cisplatin and compare it with the genotoxicity of repeated doses of halothane or isoflurane. Alteration of kidney cells DNA and peripheral blood leucocytes (PBL) harder DNA damage were found in Isoflurane exposure, indeed halothane caused genotoxicity on liver cells and brain. For the gentoxicity of cisplatin, it caused a lower genotoxicity in combination with isoflurane on peripheral blood leucocytes in comparaison with isoflurane alone [23]. Monteiro Neto et al., studied the genotoxic potential of artepillin C and its ability to reduce the chemically caused chromosome breakage or loss and the primary DNA damage using in male Swiss mice. The animals were treated by gavage with different doses of artepillin C (0.4, 0.8 and 1.6 mg kg^{-1} b.w.). For the antigenotoxicity assays, the different doses of artepillin C were administered simultaneously to methyl methanesulfonate. The results showed that artepillin C itself was not genotoxic in the mouse comet extent assays. Artepillin C at the tested doses significantly reduced DNA damage extent in liver cells induced by MMS [62]. León-Mejía et al., evaluated genotoxic effects in a population exposed to coal residues from the open-cast mine "El Cerrejón". They included in this study 100 exposed workers and 100 non-exposed control individuals [63]. These results indicate that exposure to coal mining residues may result in an increased genotoxic exposure in coal mining workers, the mean values of the Comet assay variables of the two study groups was demonstrated increased levels of DNA damage in the exposed group compared to the non-exposed control group. No difference was observed between the exposed groups executing different mining activities [63]. Villani et al., tested bull sperm treated with additional mutagens (bleomycin colchicine, diethylsulfate and mitomycin C) with the standard comet assay, mutagenisis effect of diethylsulfate was obtained with themodified assay is dose-dependent, according to their study treatment with colchicine don't causes DNA lesions [64].

Conclusion

Genotoxic damage is the most extensively studied end points because it has direct and long-lasting effects on the organism [16]. In the figure below we explain the different effects of gentoxic agents on cells (Figure 5).

Early necrosis caused by genotoxic agent (1), necrosis effect (2), DNA damages during the G0 to S phase (3), apoptosis (5), resulting in the formation of an apoptotic bodies, during the tardy apoptosis (6) or, leave to chromosomal damages, resulting in micronucleus formation through the chromosome breaks and/or disruption of mitotic apparatus (4), designated micronucleated cells to apoptosis (5 and 6) [65].

Genotoxicity tests are still the most common application of the comet assay [22]. The comet assay is a very useful for measuring DNA damage in numerous varied pathological and physiological conditions on numerous agents. The unique aspect of this method is its ability to detect damage at the level of the individual cell [17].

In this review we have tried to deal with theoretical and practical importance of comet test for the detection of DNA damage. This review

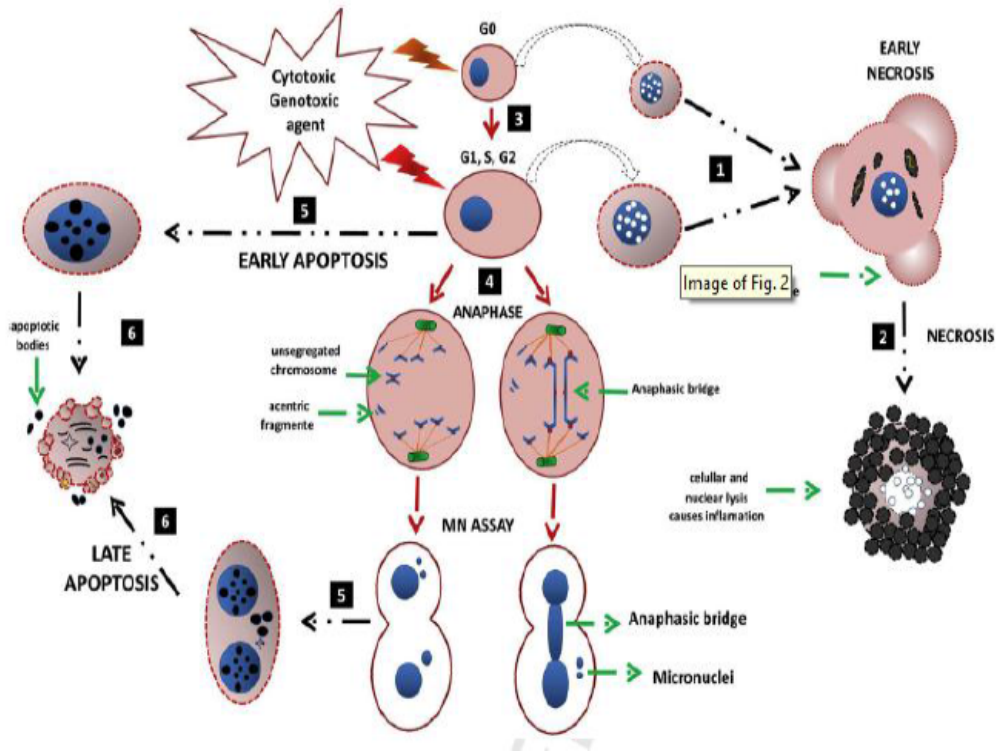

Figure 5: Different pathway of genotoxic Agent [65].

shows that the number of studies that use the comet assay for evaluation different genotoxic agent is increasing fast. In the present review, many *in vitro* studies and several *in vivo* studies have been discussed. The cellular studies show that the majority of the studied agents cause DNA strand breaks and or oxidative DNA lesions. This is not surprising considering the sensitivity of the method and the reactivity of many agents. However, interactions with the assay need further consideration and cannot be totally excluded. It is recommended to use additional methods to overcome this issue [12]. Its application in the genotoxicity testing seems to increase with the development of high throughput comet assay methods and fully automated comet analysis [66].

Disclosure of Interest

The auditors declare that they have no conflicts of interest concerning this article.

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