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Do cytotoxicity and cell death cause false positive results in the *in vitro* comet assay?

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ABSTRACT

The comet assay is used to measure DNA damage induced by chemical and physical agents. High concentrations of test agents may cause cytotoxicity or cell death, which may give rise to false positive results in the comet assay. Systematic studies on genotoxins and cytotoxins (i.e. non-genotoxic poisons) have attempted to establish a threshold of cytotoxicity or cell death by which DNA damage results measured by the comet assay could be regarded as a false positive result. Thresholds of cytotoxicity/cell death range from 20% to 50% in various publications. Curiously, a survey of the latest literature on comet assay results from cell culture studies suggests that one-third of publications did not assess cytotoxicity or cell death. We recommend that it should be mandatory to include results from at least one type of assay on cytotoxicity, cell death or cell proliferation in publications on comet assay results. A combination of cytotoxicity (or cell death) and proliferation (or colony forming efficiency assay) is preferable in actively proliferating cells because it covers more mechanisms of action. Applying a general threshold of cytotoxicity/cell death to all types of agents may not be applicable; however, 25% compared to the concurrent negative control seems to be a good starting value to avoid false positive comet assay results. Further research is needed to establish a threshold value to distinguish between true and potentially false positive genotoxic effects detected by the comet assay.

1. Introduction

The alkaline comet assay detects single and double strand breaks and alkali-labile sites when applying the standard version of the assay, altered nucleobases when using the so-called enzyme-modified comet assay, or even DNA crosslinks when applying a certain modification of the assay [1–3]. The *in vivo* version on animal tissues has been adopted as Organization for Economic Co-operation and Development (OECD) guideline test number 489 in 2014 (and updated in 2016) [4] and it is part of the test battery for the safety assessment of new medicines [5], and food and feed [6], whereas the use of the *in vitro* comet assay in regulatory toxicology is limited to being recommended to provide complementary information on genotoxicity and to elucidate the mechanism of action of chemicals and nanomaterials [7,8]. Nevertheless, it is the most used technique to assess the genotoxicity of nanomaterials [9–14]. In addition, the *in vitro* version of the comet assay is widely used to study the genotoxic hazard of chemicals and agents which are a matter of concern in environmental and occupational health research [15,16].

At high concentrations, non-genotoxic agents can cause toxic effects resulting in DNA fragmentation and cell death [17,18]. Thus, cytotoxicity and cell death may cause false positive results in some genotoxicity assays. This phenomenon has been observed in the alkaline unwinding and the alkaline elution assays [19–21]. Alkaline unwinding and alkaline elution were previously the most used assays for detection of DNA strand breaks in eukaryotic cells, although they are not used much anymore due to the popularity of the comet assay [22]. Garberg et al., in 1988, showed that excessive cell death, measured by the trypan blue (TB) dye exclusion method, was associated with elevated levels of DNA strand breaks in the alkaline unwinding assay [19]. Some years later,

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Elia et al. demonstrated that cell death-induced DNA double strand breaks produced false positive responses in the *in vitro* alkaline elution assay [20].

Most of the studies dealing with effects of cytotoxicity and cell death on DNA damage levels in the comet assay were done 20–25 years ago (described in detail below). The experiments were carried out during a period when the comet assay and cytotoxicity assays were less developed. There is a need to thoroughly reassess the effects of cytotoxicity and cell death on comet assay results and, if possible, to find the appropriate threshold of these effects to avoid obtaining false positive results in the comet assay. The effect of cytotoxicity and cell death in exposed cells can in principle affect all cells (i.e. increasing the migration of DNA in the electrophoretic field) or give rise to a subset of comets with high levels of DNA migration. The latter are described as "highly damaged cells", "ghost cells" or 'hedgehogs'. Irrespective of the synonym, these comets are characterized as having a tiny/shrunken head and a large tail. In this paper we use the term *hedgehog* to discriminate these images from comets with less DNA migration.

The aim of this review is to assess whether DNA damage measured by the comet assay is biased by concurrent cytotoxicity or cell death in cell culture studies. We provide a brief overview of the assays used to detect cytotoxicity and cell death. As a snapshot of the current state of the field, we have surveyed the use of these assays of non-genotoxic effects in the most recent papers where comet assay results are also reported. Finally, we provide perspectives for future research to fill knowledge gaps and give recommendations on the assessment and interpretation of results from cytotoxicity and cell death assays when performing the comet assay.

2. Types of cytotoxicity and cell death assays

There are different views on definitions of cell viability, cell death and cytotoxicity. For the purpose of this paper, definitions of viability, cell death, cytotoxicity, cytostasis and proliferation are applied as described in Table 1. Cytotoxicity is a measure of the potential of a substance to cause cell injury. A cytotoxic effect can compromise the viability of a cell by perturbing its metabolic or structural integrity. However, it can also be a reversible event. One such response to exposure is cytostasis, which is a toxic event characterized by inhibition of cell division and cell growth.

A coherent definition of the viability of a cell population is "the existence of structural, metabolic and, for proliferating cells, reproductive integrities essential for preservation of life" [23]. Viability is thus a measure of the percentage of living cells in a cell population without any distinction between cells that are actively dividing or quiescent [23]. Most studies in genetic toxicology entail actively proliferating cells; therefore, viability of proliferating cells is a measure of the percentage of cells in a cell population capable of cell division. However, in the case of proliferating cells exposed to cytostatic agents the term viability does not reflect the real situation; cells are structurally and metabolically viable, but they do not divide, at least during the exposure. For non-proliferating cells, the term viability reflects differentiation between living and dead cells.

Table 1
Definitions of viability, cell death, cytotoxicity, cytostasis and proliferation.

Term	Definition
Viability (non-proliferating cells)	The capacity to perform the essential metabolic processes necessary to maintain structural and functional integrity.
Viability (proliferating cells)	The capacity to perform the essential metabolic processes necessary to maintain structural and functional integrity, including proliferation.
Cell death	Irreversible condition or processes in non-viable cells, including apoptosis, autophagy and necrosis.
Cytotoxicity	Cellular injury that may be reversible or progress to cell death.
Cytostasis	Inhibition of cell proliferation.
Proliferation	Process that leads to increased number of cells by cell division.

The most common types of cell death include: (1) apoptosis that is manifested with morphologic features such as chromatin condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage, which eventually leads to the formation of small membrane-surrounded fragments (apoptotic bodies) [24]; (2) autophagy, a process of self-cannibalization, where cells degrade their own cytoplasm and organelles in lysosomes [25]; and (3) necrosis [26,27]. Cell death can be considered an ultimate cytotoxic effect and the two terms are typically used as synonyms.

2.1. Trypan Blue exclusion, live/dead and apoptosis assays

One of the earliest methods for measuring cell death was the TB exclusion assay, which was introduced more than 100 years ago [28]. It is based on the principle that viable cells have an intact cell membrane, which blocks the TB dye from entering the cells. Cells with compromised membrane integrity are stained blue. Other frequently used vital exclusion dyes are propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI). Assays based on these dyes represent a quick and inexpensive method to assess cell death, but they can also reflect cytotoxicity, resulting from damage to cell membrane integrity.

Assays that assess both live and dead cells were introduced in the 1970 s because it was clear that misclassification of cells occurred in assays that rely only on the detection of dead cells [29,30]. These live/dead assays are based on a detection method where one dye specifically stains live cells (e.g. green fluorescence) while another dye stains dead cells (e.g. red fluorescence).

There are many methods to determinate apoptotic cell death; however, describing all of them in detail is not the purpose of this paper. For the identification of apoptosis and discrimination between viable, apoptotic and necrotic cells at the same time, flow cytometry is a preferred platform. It enables rapid assessment of multiple cellular attributes at a single cell level - in particular changes in cell morphology, the presence of phosphatidylserine on cell surface, collapse of mitochondrial transmembrane potential, DNA fragmentation, and evidence of caspase activation [31].

2.2. Membrane leakage assays

A way to assess membrane barrier function in cytotoxicity/cell death assays is to measure the leakage of intracellular components such as enzymes to the cell culture medium. Lactate dehydrogenase (LDH), a stable cytosolic enzyme, is used as a marker of cytotoxicity/cell death as it rapidly leaks from cells with damaged plasma membranes. Thus, the LDH assay is based on quantifying the enzyme activity in the cell medium and can be used in two ways. First, it may be used to directly assess cytotoxicity by measuring the release of LDH from severely damaged or dead cells and, second, it may be used to count the total number of cells (by complete lysis) or to count the amount of surviving cells after removal of the dead cells [32,33]. Similarly, the glucose-6-phosphate dehydrogenase (G6PD) assay is based on leakage of this cytosolic enzyme from cells with damaged plasma membrane to the culture medium [34].

2.3. Cellular metabolism or enzymatic activity assays

A number of cytotoxicity and cell death assays rely on measuring metabolic or enzymatic activity and are nowadays widely used in combination with the comet assay. The ATP assay is a sensitive method for measurement of cell viability because ATP is generated by cellular respiration and used as energy source in multiple processes in cells. Cells with damaged membrane lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the

cytoplasm [35]. Other assays use exogenous reagents, which viable cells convert to a coloured or fluorescent product that can be detected with a plate reader. Probably the best known metabolic dye is 3-(4, 5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide (MTT) [36]; however similar dyes (MTS, XTT, and WST-1) can be used for detection of viable cells. Similarly, the Alamar Blue assay can be used to monitor viable cell number, using resazurin as a cell permeable redox indicator that is reduced to strongly fluorescent resorufin product by viable cells with active metabolism [37].

2.4. Cell proliferation and colony forming efficiency assays

There are two reliable methods for assessment of reproductive integrity, and thus the viability of proliferating cells. The proliferation assay is based on measurement of the doubling time of a cell population (counting cells usually at 24 h intervals). OECD test guideline no. 487 contains an excellent description of proliferation assays and their use in the *in vitro* micronucleus assay [38]. The clonogenic assay or colony forming efficiency assay (CFE) is based on the ability of single cells to undergo "unlimited" division and to grow into colonies [39,40]. The CFE assay can measure both survival/cell death (by comparing number of colonies in test sample plates with number of colonies in control plates) and cytostatic effect (by comparing colony size) [39,41].

2.5. Combination of assays measuring different endpoints of cellular toxicity

As single assays, most of the above-mentioned techniques cannot unequivocally discriminate between cytotoxicity and cell death in a population of cells. For instance, the TB assay is not an appropriate cytotoxicity nor cell death assay when the test compound does not induce loss of membrane integrity. Moreover, TB stains living cells with a transient non-intact membrane. Pappenheimer, in 1917, already outlined some issues concerning the use of the TB assay to distinguish between living and dead cells [28]. Other assays that are based on metabolic activity may give rise to different interpretation of the results. In the case of the MTT, for example, a 30% decrease in cellular metabolic activity can be interpreted as the presence of 30% dead cells in the cell population or that all cells are alive with a 30% reduced cellular activity. Combining assays that detect different outcomes of cytotoxicity or cell death in non-proliferating cells expands information on toxic effects of the exposure and reduces the risk of biased comet assay results.

3. Non-genotoxic effects and comet assay – a literature survey

In order to assess how toxicity and/or viability results are currently used for the evaluations of genotoxicity by the *in vitro* alkaline comet assay, we have performed a literature review on representative publications. The following search terms were used in PubMed: "comet assay [Title/Abstract] AND genotoxicity[Title/Abstract] AND ((*in vitro*[Title/Abstract]) OR (cells[Title/Abstract])). Only papers published in 2020 and 2021 were considered. From this search we retrieved 50 papers from different institutions in which the *in vitro* alkaline comet assay was performed to evaluate the genotoxicity of chemicals, extracts or nanomaterials. The institution (i.e., university, research centre, private company, public body, etc.) of the 1st author's affiliation was considered the affiliation of each paper. Papers describing effects in primary cells, including human lymphocytes, were excluded because they are not actively proliferating cells unless they are stimulated (i.e. lymphocytes). We have also excluded studies on co-culture of cells (e.g. certain 3D co-culture models) and DNA-crosslinks.

The 50 retrieved papers come from 26 countries across the world. Fig 1 outlines the observations that are gathered from these papers. Among them, 35 papers used the same concentrations and exposure times in cytotoxicity/cell death assays and comet assay experiments. Fifteen papers do not contain information on cytotoxicity and cell death or the

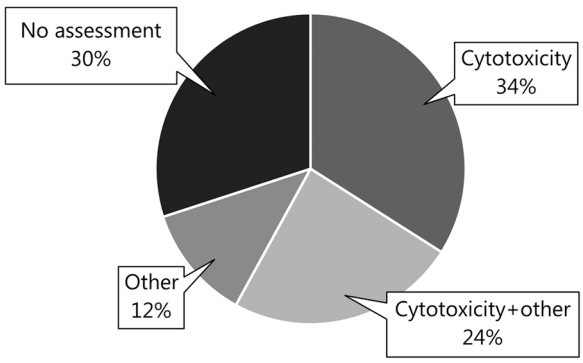

Fig 1. Distribution of 50 selected papers that have reported comet assay endpoints in 2020–2021. The groups are stratified into papers that used only cytotoxicity assays (defined as metabolic or enzymatic activity), cytotoxicity assays and other measures of non-genotoxic effects in cells (i.e. cell death, apoptosis or cell proliferation), or studies that used other techniques than metabolic/enzymatic activity to assess non-genotoxic effects. Fifteen papers (30%) did not report results from any endpoints related to cytotoxicity, cell death, apoptosis or cellular proliferation.

effect is inferred from results obtained by using other cell lines or later time-points of exposure than the ones used in the comet assay.

From the 35 papers in which comet assay data were accompanied with the cytotoxicity and cell death data, 17 used a cytotoxicity assay (e.g. MTT), 9 applied a cytotoxicity assay together with an assay to determine apoptosis, 2 used a cytotoxicity assay together with an assay to determine apoptosis and an assay to determine cell death (i.e., exclusion assay such TB or other dyes), and one used a cytotoxicity assay together with an assay to determine apoptosis and an assay to determine proliferation. Regarding the rest, 4 employed only cell death assays (i.e., exclusion assay such TB or other dyes), one used a proliferation assay and another one applied a proliferation assay together with a cell death assay.

The most used cytotoxicity assays were the ones measuring metabolic activity of the cells (i.e., MTT, MTS, XTT and similar); 25 of the papers relied on this type of assays. In 6 out of 25 papers, two different cytotoxicity assays, measuring different endpoints, were applied. Overall, the MTT is by far the most used assay, being used in 17 out of the 25 papers.

Interestingly, only 10 out of the 35 papers using cytotoxicity assays specified a cut-off to avoid false positive results in the comet assay. The cut-off varied from 20% to 60% of the cells showing cytotoxic effect. The most used cut-off was 50%, used in 3 of the papers. Regarding the TB exclusion assay, 10% and 20% of dead cells have been used as the cut-off.

4. Effects of cytotoxicity and cell death on DNA damage measured by the comet assay

4.1. Does cell death affect the comet assay?

The first systematic investigation of the effect of cell death on comet assay results was reported by Hartmann and Speit in 1997, who demonstrated that 2 h exposure to three different cytotoxins (*p*-nitrophenol, D-menthol and sodium N-laurosyl sarcosine) in V79 Chinese hamster cells or human white blood cells did not affect the level of DNA migration, despite a high prevalence of dead cells (35–75%) and reduced post-exposure plating efficiency (up to 100% reduction) [42]. "Viable" and "dead" cells were measured using a live/dead assay (i.e. fluorescein diacetate and ethidium bromide). It is worth considering that hedgehogs were excluded from the analysis by Hartmann and Speit [44]. Similar results were obtained by Hartmann et al., in 2001, who did not observe any effect of cell death (up to 43% measured by the TB

assay) on levels of DNA strand breaks in a large study using 3 h exposure of V79 cells to 75 new drug candidate compounds [43]. Henderson et al, in 1998, studied the ability of the comet assay to discriminate between genotoxic and cytotoxic compounds in TK6 cells by testing 11 compounds with different mechanisms of action, including four cytotoxins (i.e. sodium dodecyl sulphate (SDS), potassium cyanide, trypsin and cycloheximide), after 30 min exposure at 4ºC [44]. The unusual cold exposure condition was used to prevent DNA repair. Cell death was measured using the TB exclusion assay and the results led the authors to suggest a cut-off point of $<$ 25% dead cells to prevent false positive results on the comet assay.

Kiffe et al., in 2003, showed that exposure of Chinese hamster ovary K5 cells to D-menthol for 3 h did not produce DNA strand breaks in adherent cells [45]. However, in this work, a special protocol using both adherent and floating cells yielded samples with many dead cells (89%) and high levels of DNA strand breaks. These dead cells had almost all DNA in the tail (i.e. hedgehogs), which led to the suggestion that such cells should not be scored as they were not generated by primary genotoxic mechanisms of action. However, as demonstrated by Lorenzo et al., DNA damage leading to hedgehogs in cells after hydrogen peroxide exposure is quickly repaired and the disappearance of such cells is not due to further DNA degradation in the dead cells, as the cell number remain the same in the gel [46]. Above all, it demonstrates that hedgehogs cannot be regarded as dead cells (i.e. they can be primary lesions by the genotoxic agent), although dead cells can also give rise to hedgehogs.

Speit et al., in 2014, investigated the effect of the exposure to SDS and D-menthol in TK6 cells, where the scored comets were stratified into measurable images by a software system and comets that are not readily measurable using an image analysis system (i.e. hedgehogs) [47]. Fig 2 depicts the relationship between the level of cell death and the level of total DNA damage in all cells (i.e. hedgehogs + non-hedgehogs) or DNA damage without hedgehogs (i.e. measurable comets). Exposure for 2 h at the highest concentration of SDS increased the level of DNA migration (i.e. % DNA in tail) in measurable comets and the number of hedgehogs, whereas all comets were hedgehogs at 4 h exposure. On the other hand, the highest concentration of D-menthol produced virtually only hedgehogs.

Table 2 summarizes results from several published studies on effects of cell death on DNA strand breaks by non-genotoxic agents measured using dye exclusion assays, ordered by increasing maximal effect of cell death. The ordering of non-genotoxic cytotoxins in Table 2 suggests that severe cell death is associated with increased level of DNA damage by the comet assay. Segregating the results in Table 2 into compounds with maximal level of $\leq$ 45% dead cells versus compounds that produced $\geq$ 70% cell death indicates an effect of cell death (1 out of 10 versus 4 out of 8 compounds, respectively). Potassium cyanide was genotoxic in the group of compounds with low level of cell death [44]. However, potassium cyanide is a fast-acting inhibitor of cytochrome C oxidase, leading to intracellular ROS production [48]. Thus, potassium cyanide has an indirect mechanism of action of genotoxicity, which is similar to other agents that cause DNA damage by ROS production such as air pollution particles and nanomaterials [49].

Based on observations of non-genotoxic compounds, there is evidence to suggest that a high level of dead or dying cells is associated with increased level of DNA damage, measured by the comet assay. However, it is not possible using the present dataset to come close to the definition of one particular threshold where this cell death bias occurs. Based on the results in Table 2, it appears that comet assay results are not affected in samples with less than 45% dead cells.

4.2. Does apoptosis affect the comet assay?

While extensive DNA damage can cause a cell to enter apoptosis, the process of apoptosis itself is also associated with the occurrence of DNA breakage. Often, the appearance of hedgehogs has been considered to be

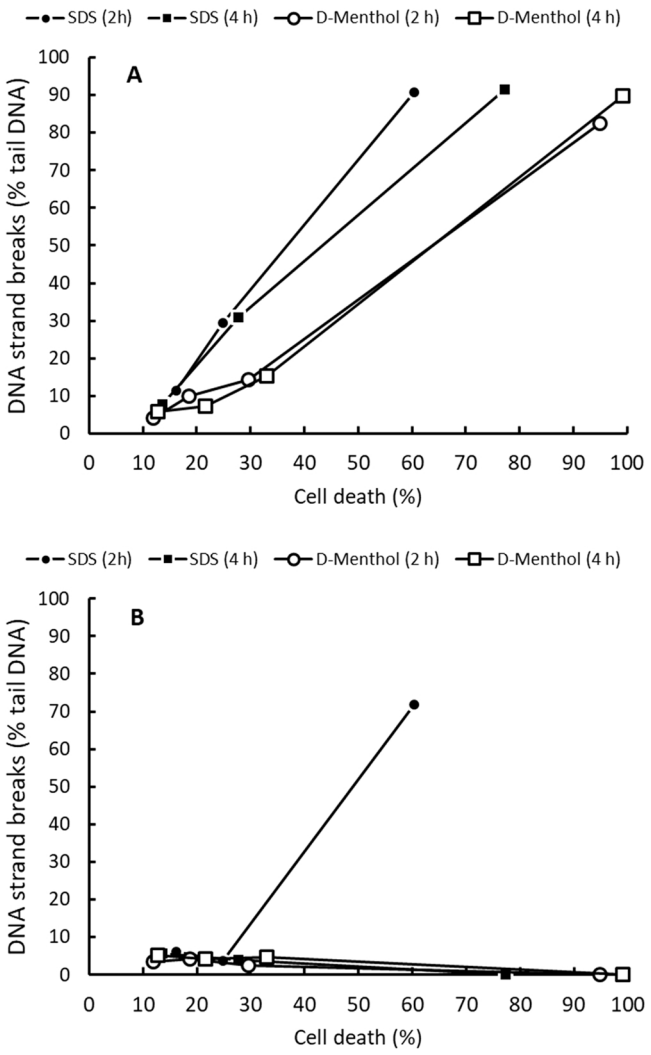

Fig 2. Relationship between cell death (i.e. Trypan Blue exclusion assay) and DNA strand breaks in TK6 cells after exposure to SDS and D-menthol for 2 or 4 h. A) Level of DNA strand breaks with hedgehogs. The level of DNA strand breaks has been calculated from information about the percentage of hedgehogs and DNA migration levels in measurable cells, using the formula as follows: Total damage = [(%Hedgehogs * 95%tail DNA) + (100% - %Hedgehogs)* (% measurable cells*%Tail DNA in measurable cells)]/100. The DNA migration value in hedgehogs is arbitrary set to 95%Tail DNA. B) Level of DNA strand breaks without hedgehogs. Original results have been published by Speit et al. [49].

an indicator of apoptosis in the comet assay. However, not all hedgehogs are apoptotic cells and not all apoptotic cells are detected as hedgehogs [42,46,50]. The small oligonucleotides of late stage apoptotic cells may disappear from the cellular area during electrophoresis in the comet assay. This may explain observations that less damaged DNA has also been observed in the comet assay in the context of apoptosis [51,52]. Godard et al., in 1999, induced apoptosis by the non-genotoxic agent staurosporine and looked at early and late apopotic stages [53]. They found Annexin-positive cells already after one hour, followed by hedgehogs in the comet assay after three hours. Late apoptotic cells (i.e. defined as Annexin-positive plus damaged cell membrane) did not correlate with the appearance of a specific pattern in the comet assay as nucleoids with highly fragmented DNA most likely disappeared from the gel during electrophoresis.

The complexity of the relationship between apoptosis and the comet assay is further increased by the fact that biomarkers of apoptosis may be transient or reversible and such characteristics of apoptotic cells may

Table 2
DNA strand breaks measured by the alkaline comet assay in cell cultures exposed to non-genotoxic compounds.

Agent	Exposure time (hours)	Cell	Cell death[a]	Effect on DNA strand breaks	Reference
Cycloheximide	0.5 (4 °C)	TK6	13% (TB)	None	[44]
Menthol	2	CHO K5	21% (PI)	None[b]	[45]
Trypsin	0.5 (4 °C)	TK6	23% (TB)	None	[44]
Glucose	4 or 24	TK6	25% (TB)	None	[84]
Tunicamycin	4 or 24	TK6	30% (TB)	None	[84]
Potassium cyanide	0.5 (4 °C)	TK6	33% (TB)	Increased	[44]
Menthol	2	WBC	35% (EtBr)[c]	None	[42]
New drug candidates (75 different compounds)	3	V79	43% (TB)	None	[43]
2.4-dinitrophenol	4 or 24	TK6	44% (TB)	None	[84]
Ethanol	4 or 24	TK6	45% (TB)	None	[84]
Triton X-100	4	TK6	70% (TB)	Increased	[85]
D-menthol	2	V79	75% (EtBr)[c]	None	[42]
SDS	0.5 (4 °C)	TK6	75% (TB)	Increased	[44]
SDS	2 or 4	TK6	80% (EtBr)	Increased	[47]
Menthol	2 or 4	TK6	80% (EtBr)	Increased	[47]
Sodium N-laurosyl sarcosine	2	V79	85% (EtBr)[c]	None	[42]
Sodium N-laurosyl sarcosine	2	WBC	89% (EtBr)[c]	None	[42]
p-nitrophenol	2	V79	97% (EtBr)[c]	None	[42]

[a] Number refers to the percentage of dead cells in the exposure group with highest concentration or the highest level of cell death by the exposure.

[b] A special protocol with collection of all cells (i.e. adherent and floating cells) showed high level of cell death (90%) and concomitant high level of DNA strand breaks.

[c] Included assessment of viable (fluorescein diacetate staining) and dead (ethidium bromide) cells. Results in the table are exposures without S9 mix. The study also included analysis of plating efficiency (5 days post exposure). The exposure resulted in reduced plating efficiency at the highest concentration.

be detected in certain methods but may not appear as damaged cells in the comet assay. Already in 2001, flow-cytometry-sorted Annexin-positive cells were shown to survive and replicate after removal of the apoptotic stimulus [54]. Since then, processes such as anastasis and so-called failed apoptosis have been described, which can rescue apoptotic cells [55,56]. It has been argued that fragmentation into oligonucleotides may be too extensive to be repaired, and this or the activation of caspases may be the point of no return from apoptosis. But a reversal even after late stages of apoptosis has been observed [57]. An increased frequency of cells with micronuclei has been found in cells after reversal of apoptosis [58], but such cells with genomic damage expressed as micronuclei are not necessarily detectable as positive in the comet assay.

Based on the current literature, researchers should be aware that - while the comet assay cannot be used for apoptosis detection - its results can be influenced by the occurrence of apoptosis.

4.3. Does cytotoxicity affect the comet assay?

In principle, cytotoxicity is reversible if the exposure is lifted from the cells, although very high doses or long duration of exposure increase the progression to apoptosis or necrosis. Nowadays, cytotoxicity is typically measured by high through-put assays, which are available as commercial kits. These are typically carried out at a fixed time-point (e.g., 24 h), rather than multiple time-points to monitor the development of cytotoxicity. In addition, the cytotoxicity assays serve to set the maximal concentration where primary outcomes of the experiments are not biased by cytotoxicity (i.e., as false positive findings). To the best of our knowledge, there are no large-scale studies that have assessed associations between responses on metabolic/enzymatic activity and DNA damage measured by the comet assay. However, an early study on primary rat hepatocytes assessed the association between cytotoxicity, measured by the MTT and intracellular ATP assays, and DNA strand breaks measured by the alkaline elution assay [21]. Fig 3 shows the association between the level of cytotoxicity (MTT and intracellular ATP assays) and DNA damage in rat hepatocytes. As can be seen, substantial increases in DNA damage were first observed at approximately 70% cytotoxicity in the MTT and intracellular ATP assays [21]. A later study, using the intracellular ATP assay, confirmed an association between high level of cytotoxicity and DNA damage measured by the alkaline elution assay in rat hepatocytes using 3 h exposure [59].

Based on the current knowledge, at least very high levels of cytotoxicity are associated with artificial generation of DNA damage in cell cultures. Cytotoxicity assays also detect cell death and it should be kept in mind that cell death cannot be excluded when cytotoxicity assays are performed.

4.4. Is there a threshold of cytotoxicity or cell death that biases comet assay results?

Curiously, our literature review (see section 3) showed that only a few publications actually mention the use of a threshold level of cytotoxicity or cell death. Nevertheless, thresholds between 20% and 30% cellular cytotoxicity or cell death are most commonly used. The first comprehensive guideline for the *in vitro* comet assay recommended a threshold of 30% for cytotoxicity or cell death, although the expert panel was not able to identify the optimal methods for the assessment of these [60]. The testing guideline referenced an earlier publication with original results on cytotoxins and a review of mainly the concordance between genotoxicity of chemical compounds in cell cultures and animal tissues, both of which references mentioned a cut-off of 25% dead cells [44,61]. The threshold that has been determined for the *in vitro* skin comet assay is 50% of cytotoxicity (50% reduction of ATP release, or doubling of adenylate kinase release) [62]. A threshold of 50% dead cells has been recommended in one *in vitro* comet assay protocol [63]. Another protocol only mentions the need for cytotoxicity/viability tests to rule out false positive results in the comet assay, but does not recommend a specific threshold of cytotoxicity and cell death [64].

Other *in vitro* assays of genotoxic endpoints on irreversible changes, such as mutations, require actively dividing cells and therefore use cell proliferation assays for establishing the upper level of exposure. Target levels of reduced proliferation are 50% for the chromosomal aberration test and 55% for the micronucleus assay in cell cultures [65]. Measures of proliferation and CFE have not been used systematically in validation studies on the comet assay but have been applied in some studies of nanogenotoxicity [41, 66–68] with recommendation of a cut-off of 20% reduced proliferation or CFE because it could be used to discriminate between genotoxic and non-genotoxic nanomaterials. These studies recommend using two types of cytotoxicity assays, namely proliferation or CFE as well as TB or metabolic activity assay such as Alamar Blue.

Based on the current knowledge, there is no consensus on the threshold of non-genotoxic effects that could be applied to avoid false positive results in the comet assay. The studies indicate that the

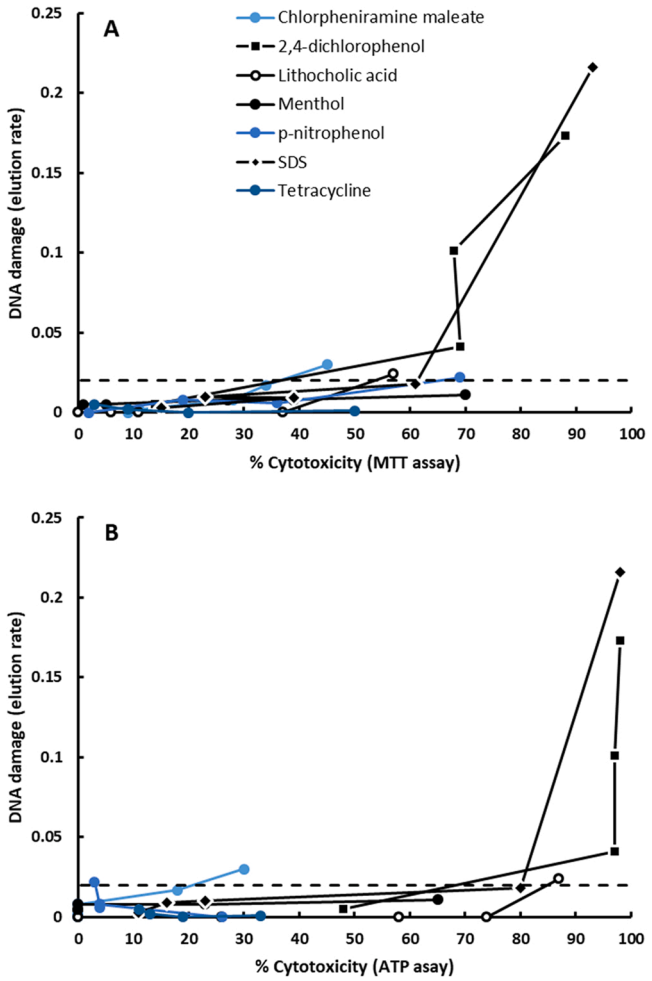

Fig 3. Relationship between cytotoxicity measured by the MTT (A) or intracellular ATP level (B) assays and DNA strand breaks (alkaline elution) in rat hepatocytes at 3 h exposure to non-genotoxic and non-carcinogenic agents. Original results includes 28 test compounds; the compounds in the graph are those that produced more than 50% cytotoxicity or cell death in at least one assay (chlorpheniramine maleate, 2,4-dichlorophenol, lithocholic acid, menthol, *p*-nitrophenol, phenfomin, sodium dodecyl sulphate and tetracycline). Values on the y-axis is the induced slope (i.e. net difference in elution rate between the treatment and negative control slope). The authors set 0.020 as criteria for a genotoxic response (dotted line). In comparison, elevated elution rates were observed at the highest concentration of 4-nitroquinoline-*N*-oxide produced (0.294), etoposide (0.329) and methyl methane sulfonate (0.772), whereas there were no appreciable effect on cytotoxicity markers. Original results have been published by Storer et al. [21]. In the original publication, the results are reported as "Cytotoxicity (% of Control)", where 100% refers to the background level in unexposed cells and increased levels of cytotoxicity corresponds to lower % values. We have inverted the scale in figure, so 0% cytotoxicity refers to the background level in unexposed cells and increased cytotoxicity corresponds higher percent values.

threshold could be between 20% and 50% response in cytotoxicity, cell death, proliferation and CFE assays. Using one fixed low threshold (e.g. 20% dead cells) might be too strict and might in some cases result in false negative findings, while a much less conservative cut-off (e.g. 50% dead cells) may lead to false positive findings.

5. Final remarks and suggestions

Recently, there has been a surge of articles describing the comet assay procedure for both DNA damage and repair, as well as recommendations for minimal information on procedures and results that need to be reported in papers [69–71]. In addition, a number of papers on technical issues and recommendations to prepare positive assay controls and cryopreservation of cells have been published [72–74]. However, technical issues related to the effect of cytotoxicity and cell death on comet assay endpoints have not been systematically assessed yet.

Our literature review has demonstrated that there does not appear to be a standard practice to assess cytotoxicity, cell death, apoptosis, proliferation or CFE in studies using the comet assay as shown in the brief literature survey performed within this paper (Fig 1). We recommend that it is mandatory to report results from at least one toxicity endpoint in publications on comet assay endpoints. Comet assay results from *in vitro* experiments should be accompanied by assessment of cytotoxicity, cell death, proliferation or CFE. We also recommend to use two endpoints whenever possible, namely different cytotoxicity/cell death or proliferation/CFE endpoints to increase the certainty of correctly assessed toxicity of the exposure in the cell culture. As the number of endpoints increases, so does the possibility of conflicting results between assays on the same endpoint or different endpoints. Fig 4 outlines a flow chart of questions and decisions to make in case results from different cytotoxicity, cell death or proliferation assays differ. For actively proliferating cells it is preferable to assess one cytotoxicity/cell death marker and proliferation/CFE. For non-proliferating cells, it is preferable to assess two cytotoxicity or cell death markers. In addition, determining cytotoxicity after the exposure period and cell death (or proliferation) after a post-treatment incubation is valuable for the interpretation of the consequence of DNA damage. An apoptosis marker is valuable in some cases, but this endpoint should not be mandatory since it requires special techniques (e.g. flow-cytometry). Selecting specific endpoints and assays is a case-by-case decision.

It is desirable to establish a threshold of cytotoxicity and cell death, which does not produce false positive results by the comet assay. However, the current literature does not indicate that a consensus can be

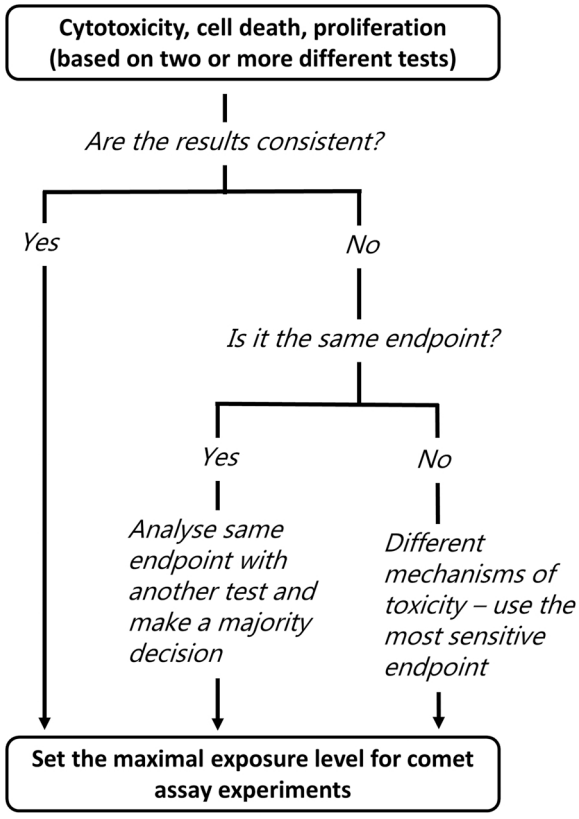

Fig 4. Flow chart of decision tree by which cytotoxicity, cell death or proliferation assays are used to select the maximal exposure level in the *in vitro* comet assay.

reached on a specific threshold. At present, either interpreting the concentration-response relationship or using a cut-off in cytotoxicity, cell death or proliferation assays seem to be applicable procedures. In a specific study, thresholds of unacceptably high cytotoxicity, cell death or reduced proliferation may be dictated by earlier experience (or personal preferences), knowledge of unique adverse outcome in specific cell types or higher risk of bias due to cytotoxicity in other assays than the comet assay. Although it should not be considered as a definitive threshold, 25% cytotoxicity is a good starting value for flagging concerns about false positive comet assay results for chemicals and nanomaterials with unknown mechanism of action. However, it should be noted that values in different cytotoxicity assays may not be equivalent (e.g. 25% increase in LDH leakage from cells is not equivalent to 25% reduced metabolic activity in the MTT assay). Likewise, the sensitivity of cytotoxicity assays may depend on type of compound and the mechanism of action.

Based on our review there are a number of important knowledge gaps that require further experiments to be solved. It is recommended that further validation studies be carried out to assess the effect of cytotoxicity and cell death on DNA damage levels in the comet assay. Preferably, this is explored using state of the art techniques. These should include the most used assays for cytotoxicity, cell death, apoptosis, proliferation and CFE. In addition, it is recommended to incorporate ring trials into such validation studies to assess inter-laboratory heterogeneity in test results and to increase the general applicability of the findings. Inspiration to validation trials may come from works on harmonization of procedures in nanotoxicology, which have entailed a number of inter-laboratory trials on cytotoxicity assays, including LDH, WST-1, and MTS assays that have demonstrated substantial variation in maximal values of cytotoxicity or concentration-response relationships in cell cultures after exposure to nanomaterials [75–79]. Reasons for these inter-laboratory differences in cytotoxicity responses are not elucidated, although they may be due to different clones of cells, cell cultures and exposure conditions. The variation in cytotoxicity response should be taken into consideration when assessing the effect of cytotoxicity on DNA damage measured by the comet assay. Inter-laboratory validation trials on comet assay experiments have also shown relatively large differences in DNA damage values when identical samples were analysed by either laboratory-specific or standardized procedure in different laboratories [80–83]. Thus, inter-laboratory variation in cytotoxicity and genotoxicity responses should be taken into account if results from one study are generalized to all laboratories.

In conclusion, this review demonstrates that high levels of cytotoxicity and cell death can affect DNA migration in the comet assay. However, there is no consensus for a threshold, although avoiding more than 25% cytotoxicity, cell death and decreased proliferation rate is often considered to avoid false positive comet assay results. There is a need for further validation studies to establish threshold values to distinguish between a true and potentially false positive genotoxic effect detected by the comet assay.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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