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Coupling cytotoxicity biomarkers with DNA damage assessment in TK6 human lymphoblast cells

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ABSTRACT

There is considerable discussion within the scientific community as to the appropriate measures of cytotoxicity to use when deciding on the maximum concentration of a substance to test *in vitro* for its ability to induce DNA damage using the Comet assay. Conventional cytotoxicity assessment methods, such as trypan blue dye exclusion or relative cell number (cell counts) may not be the most biologically relevant measurement for cytotoxicity in this assay. Thus, we evaluated for decreased levels of adenosine triphosphate (ATP) and activation of Caspase-3/7 as well as relative cell number and trypan blue exclusion in order to understand the correlation among test compound concentration, cytotoxicity and genotoxicity outcomes in the Comet assay. We tested two non-genotoxic and non-cytotoxic compounds (D-glucose and ethanol), two non-genotoxic but cytotoxic compounds (2,4-dichlorophenol and tunicamycin) and four genotoxic and cytotoxic compounds (methyl methanesulfonate, ethyl methanesulfonate, etoposide and 4-nitroquinoline-N-oxide) in TK6 human lymphoblast cells. Our data show that measuring ATP and Caspase-3/7 levels provides more rapid and perhaps more biologically relevant measures of cytotoxicity compared with trypan blue dye exclusion and relative cell number. Furthermore, incorporating these two assays into the Comet assay also provided insight on the cytotoxic mode of action of the chemicals tested. By extrapolation, such assays may also be useful in other *in vitro* genotoxicity assays.

1. Introduction

In *in vitro* mammalian cell-based genotoxicity tests, the maximum concentration of a test substance is determined by cytotoxicity, unless limited by solubility in solvent or treatment medium, or having achieved the required maximum concentrations (5 mg/mL or 10 mM) [1]. A variety of methods have been used to measure cytotoxicity in *in vitro* genotoxicity assays. These include cell growth inhibition, culture confluency, mitotic index (MI), cloning efficiency (CE), binucleate index (BI) and trypan blue dye exclusion [2,3]. However, depending on the method used to measure cytotoxicity, the maximum concentration of a test substance required for an adequate test can vary greatly within and across different cell types [4]. Moreover, many of these measurements usually require manual scoring and therefore are labor-intensive and time-consuming. Hence, developing new methods for measuring cytotoxicity that are reliable, quicker and have higher throughput would be of great significance in genotoxicity studies.

The adenosine 5′-triphosphate (ATP) bioluminescence assay [5] is based on a simple luciferin–luciferase reaction that measures bioluminescent signal proportional to the ATP content within the cells. Since ATP and its phosphate bonds are the basic components of energy exchange in many biological systems and the quantity of ATP within each type of cells is reasonably uniform, this assay provides a direct measurement of the number of metabolically active (viable) cells present. The ATP assay has been used in detecting low-level bacterial contamination [6], in drug discovery screening [7,8], in studying mechanisms of anti-cancer drugs on malignant cell growth [9], and as a quantitative high throughput screening assay for the cytotoxicity of large libraries of environmentally important substances [10]. This assay has also been evaluated as a measurement of cytotoxicity in the chromosomal aberration (CA) [11] and the alkaline elution assays [12–14].

Chemical-induced cell death can occur via necrosis or apoptosis (programmed cell death), which possesses distinct morphological and biochemical characteristics. While necrosis usually refers to cell death under extreme conditions that physically damage the cells, apoptosis is a more complex, strictly regulated process that involves a cascade of signal transduction steps. Caspases, a family of cysteine aspartic acid-specific proteases, play an essential role in apoptosis by activating downstream effectors and cleaving target proteins, which culminate in cell death [15]. Particularly, Caspase-3 and -7, emerging signals from both internal apoptosis

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pathways such as mitochondrial and endoplasmic reticulum (ER) pathways, and external stimuli through death receptor pathways, serve as executioner caspases that directly lead to protein cleavage and cell death [16,17]. The Caspase-3/7 assay contains a luminogenic Caspase-3/7 substrate, which is routinely suppressed by the tetrapeptide sequence Asp-Glu-Val-Asp (DEVD). When cleaved by active Caspase-3 or -7, the released substrate can react with luciferase and produce luminescence. Given its high sensitivity and reproducibility, this assay has been frequently utilized in studying various biological processes involving apoptosis [18,19]. Combining the Caspase assay with the genetic toxicology tests will not only provide an assessment of cytotoxicity, but might also shed light on the mode of action (MOA) of the test chemicals.

We evaluated the use of these two cytotoxicity assays, ATP bioluminescence and Caspase-3/7 activation, in the *in vitro* Comet assay, a widely used genotoxicity test to detect the ability of test substances to induce DNA damage (single-strand breaks, double-strand breaks and alkali-labile sites) [20,21]. Although other methods are being considered, cell viability determined by trypan blue dye exclusion assay has been used as the primary cytotoxicity measurement in this assay. It has been reported that scoring only samples with viability >70% in the Comet assay may set an appropriate criterion to minimize false positive results due to excessive cytotoxicity [22]. In contrast, approximately 50% of relative cell number is commonly required to determine the maximum concentration of a test substance to use in *in vitro* CA or micronucleus (MN) tests. Therefore, we compared ATP and Caspase-3/7 activation assays and these two more traditional methods, trypan blue dye exclusion assay and relative cell number, concurrently conducted with the Comet assay, and tested representatives of three classes of genotoxic/cytotoxic chemicals: non-genotoxic and non-cytotoxic (D-glucose and ethanol); non-genotoxic but cytotoxic (2,4-dichlorophenol (2,4-DCP) and tunicamycin) and genotoxic and genotoxic (methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), etoposide and 4-nitroquinoline-N-oxide (4-NQO). We conducted the study using two treatment/sampling times (4 and 24 h) in a human lymphoblast cell line, TK6, because although the 4-h treatment time is being considered for the *in vitro* Comet assay validation study organized by Japanese Center for the Validation of Alternative Methods (JaCVAM), we sought to determine whether including the 24-h treatment can capture DNA damage from slow-acting chemicals which may not be detected using the earlier time point. Our results from testing of various classes of compounds indicate that trypan blue dye exclusion assay appears to be the least biologically relevant method among the cytotoxicity endpoints evaluated with the *in vitro* Comet assay. The combination of the two biomarker-based assays provides a reliable assessment of cytotoxicity and could potentially be used to determine the maximum concentration to evaluate for genotoxicity in *in vitro* assays. In addition, the 4-h treatment protocol appears to be more appropriate than the 24-h treatment protocol for detecting DNA damage in *in vitro* Comet assay.

2. Materials and methods

2.1. Test chemicals

D-Glucose (Chemical Abstracts Service (CAS) Registration No. 50-99-7) and ethanol (CAS No. 64-17-5) were obtained from J.T. Baker (Phillipsburg, NJ). Tunicamycin (CAS No. 11089-65-9), 2,4-dichlorophenol (CAS No. 120-83-2), methyl methanesulfonate (CAS No. 66-27-3), ethyl methanesulfonate (CAS No. 62-50-0), etoposide (CAS No. 33419-42-0) and 4-nitroquinoline-N-oxide (CAS No. 56-57-5) were obtained from Sigma–Aldrich (St. Louis, MO). All chemicals were dissolved in dimethyl sulfoxide (DMSO) (J.T. Baker, Phillipsburg, NJ) and tested up to 5 mg/mL or 10 mM, whichever was lower, unless limited by solubility, excessive cytotoxicity or high percentage of 'clouds' (>30%) (or 'hedgehog', defined as cells without a discernable head but with a large diffuse tail). Top concentrations selected for the evaluation of DNA damage in the Comet assay based on various cytotoxicity measurements are discussed in Sections 3 and 4.

2.2. Cell culture and treatment with test chemicals

The human lymphoblastoid cell line TK6 was acquired from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (ATCC), 1% sodium pyruvate (Gibco-Invitrogen) and 1% penicillin–streptomycin (Gibco-Invitrogen), at 37 °C in 5% CO_2. Cells were seeded at 200,000 cells/mL and cell density did not exceed 1.2 million/mL during the treatment. Cells were treated with at least four concentrations of each chemical and the solvent control for 4 or 24 h to determine short- and long-term effects. All chemicals were tested in the absence of any exogenous metabolizing system since these chemicals were chosen to represent different cytotoxic and genotoxic characteristics and do not require metabolic activation to detect genotoxicity. At harvest, cells were centrifuged, washed once, and resuspended in cold Hanks Balanced Salt Solution (HBSS) (Gibco-Invitrogen). The cell suspensions were then used to determine the cell number, cell viability and DNA damage. ATP bioluminescent assay and Caspase-3/7 activation assay used unwashed cells. Experiments were performed in triplicates for each concentration.

2.3. Comet assay

The Comet assay was performed as described by Singh et al. [20] with modifications by Escobar et al. [23]. Briefly, cell suspensions (5–10 μL) were mixed with low melting point agarose (0.5%) (Fisher Scientific, Pittsburgh, PA) and applied to a superfrosted glass microscope slide (Fisher Scientific), previously coated with 1% normal melting point agarose (Fisher Scientific). The gel was allowed to solidify at 4 °C for approximately 15 min. The slides were then submerged in a cold lysis solution (2.5 M NaCl (Fisher Scientific), 100 mM EDTA (Sigma), 10 mM Trizma base (Sigma), 1% Triton X-100 (Sigma), and 10% DMSO, pH 10), and kept at 4 °C for at least 24 h. Slides were removed from lysis solution and then submerged for 20 min in an alkaline buffer containing 300 mM NaOH and 1 mM EDTA, pH 13, to allow DNA unwinding. Electrophoresis was performed in the same alkaline buffer at 0.7 V/cm and 300 mA for 30 min at room temperature. After electrophoresis, the slides were placed in neutralization buffer containing 0.4 M Trizma base at pH 7.5 for 5 min and then dipped in 100% ethanol for at least 5 min. The slides were air dried and stored at room temperature until analysis. For scoring, the slides were coded, stained with SYBR Gold™ (Molecular Probes-Invitrogen, Eugene, OR), and blindly examined under a 20× objective on a fluorescence microscope using FITC filters (490 nm excitation/520 nm emission). The images of the comets were analyzed using the Comet V™ v.4.1 software system (Perspective Instruments, Liverpool, UK). DNA damage was quantified by determining the percentage of DNA in the tail (% tail intensity). Fifty randomly selected cells per slide were scored for the cell culture experiments, and a total of 100 cells were scored for each sample. The frequency of 'clouds' was determined based on visual scoring of 100 cells per sample.

2.4. Relative cell number and trypan blue dye exclusion assay

Cell counts were determined by a Coulter Counter (Beckman Counter, CA) according to the manufacturer's directions. Relative cell number was expressed as a percentage of the cell number relative to the solvent control. Viability was examined by the trypan blue dye exclusion assay. Basically, cell suspensions were mixed with trypan blue (0.4% in water) (Gibco-Invitrogen) at a ratio of 1:1 and immediately transferred to a hemacytometer. The number of viable cells (not stained) and dead cells (stained) was counted under a microscope. Cells in all four corner squares on the hemacytometer were counted and absolute viability was calculated as a percentage of viable cells against total cells.

2.5. ATP cytotoxicity assay and Caspase-3/7 activation assay

The ATP determination kit and Caspase-3/7 activation kit were obtained from Promega (Madison, WI). The two assays were performed according to the manufacturer's protocols. Essentially, 100 μL of cell suspension from each sample was mixed with 100 μL of reconstituted reagent and the mixture was incubated at room temperature for 10 (ATP assay) or 30 min (Caspase-3/7 assay). The produced bioluminescence signal was measured in a Tecan Safire^{2}™ spectrophotometer (Tecan, Männedorf, Switzerland). ATP cytotoxicity was normalized as a percentage of the solvent control, but not corrected for cell number. Caspase-3/7 activation was expressed as a fold change of the solvent control.

2.6. Statistics

For DNA damage in the Comet assay, statistical analysis was performed using % tail intensity in order to determine if the tested compound induced an increase in the level of DNA damage. Initially, the % tail intensity data distribution was tested for normality, which was followed by a one-way ANOVA with Dunnett's correction ($p < 0.05$). A linear regression analysis was also performed to determine the existence of a concentration-dependent response. Chemicals were judged to be positive in the Comet assay if one or more concentrations were statistically elevated relative to the solvent control and a concentration-dependent increase in DNA damage was observed. Chemicals were judged to be negative in the Comet assay if no statistically

significant increase in DNA damage relative to the solvent control or no evidence of dose response are observed.

For ATP and Caspase-3/7 activation assays, one-way ANOVA with Dunnett's correction was used to determine the significant effect compared to the solvent control ($p < 0.05$). For relative cell number and trypan blue, statistical analysis was not used; instead, cut-off values of approximately 50% (relative cell number) and 70% (viability) were used, respectively, to select top concentrations for the Comet assay.

3. Results

3.1. Non-genotoxic and non-cytotoxic chemicals: D-glucose and ethanol

D-Glucose was tested at 225, 450, 900 and 1800 μg/mL (10 mM) and results of each cytotoxicity endpoint and the Comet assay are shown in Fig. 1 and Table 1. At the end of the 4-h treatment, no significant cytotoxicity was observed at any of the concentrations tested by all the cytotoxicity endpoints except for the trypan blue dye exclusion assay, in which cell viability was reduced to 63% at 900 μg/mL (Fig. 1A and Table 1). The reduction in viability was unexpected since the cells appeared to be healthy as indicated by other cytotoxicity endpoints. At 24 h, no cytotoxicity was detected by any method (Fig. 1B and Table 1). Also, no induction of Caspase-3/7 activation was observed at either sampling time (Fig. 1A, B and Table 1). Based on the trypan blue dye exclusion test data, the highest concentration to test should be 450 μg/mL at 4 h while based on any other cytotoxicity endpoint, it should be 1800 μg/mL at both 4 and 24 h. In our study, D-glucose was tested in the Comet assay at concentrations up to 1800 μg/mL at both 4 and 24 h (see Section 4 and Table 4). The Comet assay was negative for induction of DNA damage at either time point (Fig. 1C, D and Table 1).

Ethanol was tested at 56.25, 112.5, 225 and 450 μg/mL (10 mM) (Fig. 2 and Table 1). Similar to D-glucose treatment, only the trypan blue assay showed any evidence of cytotoxicity, with a decrease in viability (<70%) at the highest concentration (450 μg/mL) at 4 h and the top two highest concentrations (225 and 450 μg/mL) at 24 h (Fig. 2A, B and Table 1). Relative cell number and ATP were not altered substantially at any tested concentration at either time point. In addition, Caspase-3/7 was not activated in response to ethanol treatment at 24 h (Caspase-3/7 activation assay was not conducted at 4 h). Therefore, based on viability, the top concentration selected for the Comet assay should be 225 μg/mL at 4 h and 112.5 μg/mL at 24 h and, based on relative cell number or ATP/Caspase levels, 450 μg/mL at both sampling times (see Section 4 and Table 4). In our study, cells were exposed to 450 μg/mL and under these conditions, DNA damage was not increased at either sampling time (Fig. 2C, D and Table 1).

From these results, we determined that D-glucose and ethanol are non-genotoxic and, with the exception of the trypan blue dye exclusion test, non-cytotoxic at the concentrations tested. Among the endpoints used here, the trypan blue assay appears to be a less relevant measure of cytotoxicity since the treated cells appeared to be healthy reflected by other cytotoxicity endpoints and using higher concentrations did not result in a positive Comet assay response.

3.2. Non-genotoxic but cytotoxic chemicals: 2,4-DCP and tunicamycin

2,4-DCP was tested at 5, 10, 20 and 40 μg/mL [24] (Fig. 3 and Table 2). The test concentrations were selected according to a preliminary study where excessive cytotoxicity was observed at concentrations above 40 μg/mL (data not shown). At 4 h, relative cell number was decreased at concentrations of 10 μg/mL and above; and at 40 μg/mL relative cell number was reduced to 57%. Trypan blue determined viability only showed a significant reduction at the highest concentration tested. The ATP levels were not significantly changed at any tested concentration. Caspase-3/7 activation was observed at the highest two concentrations (Fig. 3A and Table 2). At 4 h, based on ATP/Caspase, trypan blue, and relative cell number, 10, 20 and 40 μg/mL, respectively, should be selected as the highest concentration to test in the Comet assay (see Section 4 and Table 4). At 24 h, relative cell number and viability were decreased to 33% and 57%, respectively, at 40 μg/mL. Notably, the highest two concentrations of 2,4-DCP induced a significant decrease in ATP levels and Caspase-3/7 acti-

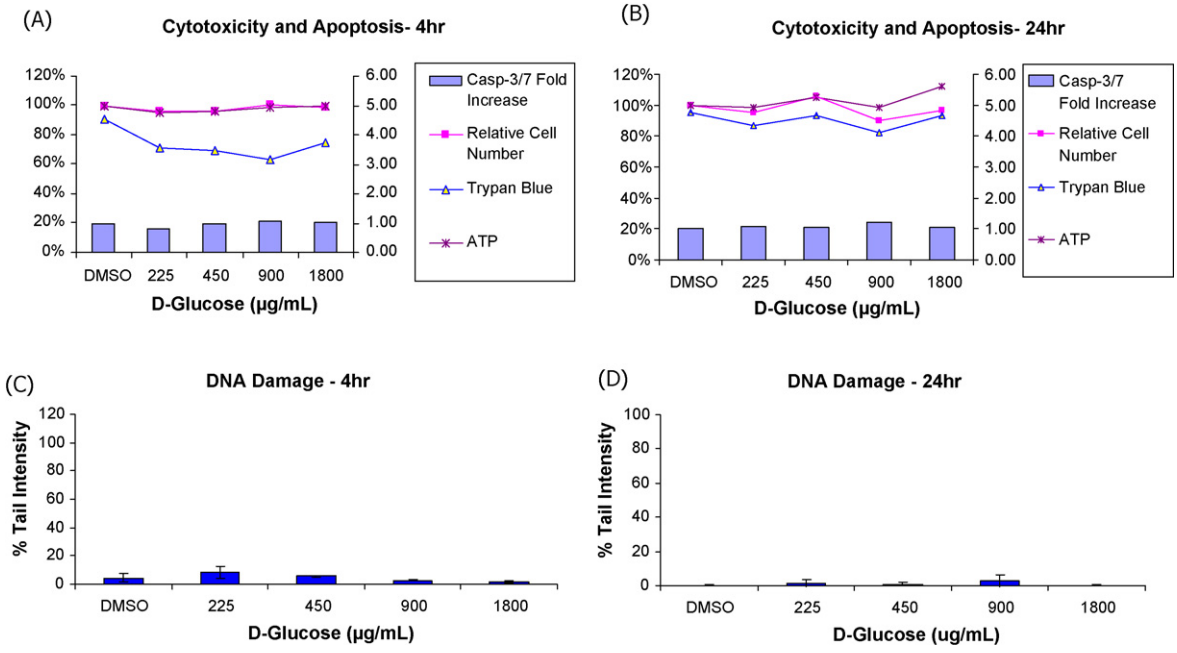

Fig. 1. Cytotoxicity and DNA damage induced by D-glucose treatment. TK6 cells were treated with D-glucose for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

Table 1
DNA damage, cytotoxicity and apoptosis induced by non-genotoxic and non-cytotoxic chemicals.

Chemical	Treatment (h)	Concentration (μg/mL)	% Tail intensity [mean (SD)][a]	% Clouds[b]	Relative cell number (%)[c]	Absolute viability (%)[d]	Relative ATP activity (%) [mean (SD)][e]	Relative Caspase-3/7 activation [mean (SD)][f]
D-Glucose	4	**0**	4.6 (3.1)	2	100	91	100 (4)	1.00 (0.04)
		225	8.4 (4.2)	2	96	71	95 (7)	0.78 (0.03)
		450	5.7 (0.5)	3	96	**69**	96 (3)	0.98 (0.07)
		900	2.9 (0.7)	2	100	63	98 (5)	1.05 (0.07)
		1800	1.7 (1.1)	1	**99**	75	**99 (4)**	**1.04 (0.04)**
	24	**0**	0.3 (0.3)	2	100	95	100 (6)	1.00 (0.04)
		225	1.4 (2.0)	3	95	87	99 (2)	1.07 (0.01)
		450	1.0 (0.8)	4	105	93	105 (1)	1.03 (0.02)
		900	2.8 (3.4)	3	90	82	98 (5)	1.22 (0.13)
		1800	0.3 (0.3)	1	**97**	**93**	**112 (1)**	**1.05 (0.06)**
Ethanol	4	**0**	5.0 (2.0)	3	100	84	100 (6)	NA
		56.25	4.1 (1.8)	4	91	74	87 (5)	NA
		112.5	4.0 (0.4)	3	91	80	99 (5)	NA
		225	4.3 (2.5)	0	92	**77**	99 (5)	NA
		450	2.8 (0.1)	0	**89**	63	**99 (1)**	NA
	24	**0**	4.2 (3.8)	4	100	89	100 (1)	1.00 (0.04)
		56.25	4.1 (0.8)	3	98	91	104 (9)	0.94 (0.12)
		112.5	6.3 (6.2)	8	92	**79**	107 (6)	0.92 (0.05)
		225	5.8 (1.9)	1	86	64	102 (3)	1.04 (0.11)
		450	2.2 (0.4)	0	**82**	58	**102 (7)**	**0.86 (0.03)**

NA, not available. Bold values indicate the top concentrations selected for evaluation in the Comet assay based on each endpoint.

[a] DNA damage is represented by the percentage of DNA fragments present in the Comet tail (% tail intensity). Each point represents the mean of medians of three replicates and in parentheses the standard deviation.

[b] Percentage of 'clouds' is represented by the percentage of highly damaged cells without a discernable head but with a large diffuse tail. Each point represents the mean of three replicates at each concentration.

[c] Relative cell number is represented by the percentage of the mean of the cell numbers of three replicates at each concentration relative to the mean of the cell numbers of the three solvent control replicates.

[d] Absolute viability is represented by the percentage of viable cells against total cells determined by the trypan blue dye exclusion assay. Each point represents the mean of three replicates.

[e] Relative ATP activity is represented by the percentage of the mean of the ATP amount in three replicates at each concentration relative to the mean of the ATP amount in the three solvent control replicates and in parentheses the standard deviation.

[f] Relative Caspase-3/7 activation is represented by the fold change of the mean of the activated Caspase-3/7 in three replicates at each concentration relative to the mean of the activated Caspase-3/7 in the three solvent control replicates and in parentheses the standard deviation.

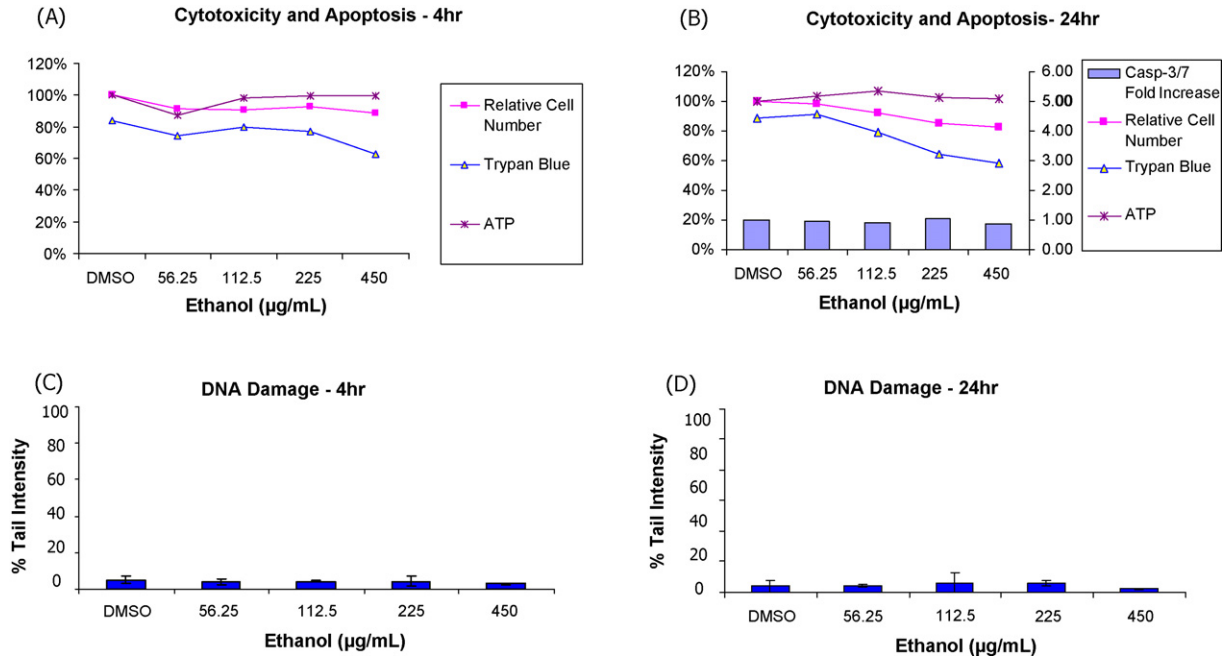

Fig. 2. Cytotoxicity and DNA damage induced by ethanol treatment. TK6 cells were treated with ethanol for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

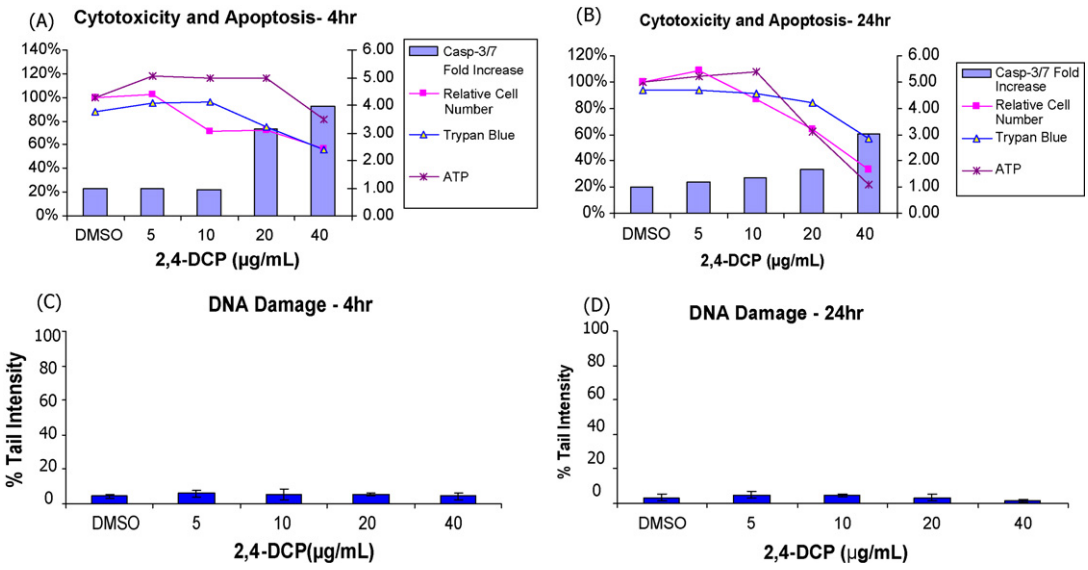

Fig. 3. Cytotoxicity and DNA damage induced by 2,4-DCP treatment. TK6 cells were treated with 2,4-DCP for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

vation (Fig. 3B and Table 2). Based on our criteria, 20 μg/mL should be selected as the top concentration based on viability or relative cell number, and 10 μg/mL should be chosen based on ATP/Caspase at 24 h (see Section 4 and Table 4). However, even at the highest concentration tested, no increase in DNA damage was detected in the Comet assay at both time points (Fig. 3C, D and Table 3).

Tunicamycin was tested at concentrations up to 200 μg/mL [25]. However, at 100 and 200 μg/mL, the 100× dose formulations precipitated in the media, and the 50 μg/mL concentration resulted

Table 2
DNA damage, cytotoxicity and apoptosis induced by non-genotoxic but cytotoxic chemicals.

Chemical	Treatment (h)	Concentration (μg/mL)	% Tail intensity [mean (SD)][a]	% Clouds[b]	Relative cell number (%)[c]	Absolute viability (%)[d]	Relative ATP activity (%) [mean (SD)][e]	Relative Caspase-3/7 activation [mean (SD)][f]
2,4-DCP	4	**0**	4.5 (1.2)	4	100	88	100 (20)	1.00 (0.03)
		5	5.9 (1.8)	2	102	95	118 (34)	1.00 (0.14)
		10	5.7 (3.0)	1	72	96	117 (25)	**0.93 (0.04)**
		20	5.5 (1.0)	7	73	**75**	117 (6)	3.15* (0.81)
		40	4.3 (1.8)	20	**57**	56	**82 (14)**	3.95* (0.29)
	24	**0**	3.5 (2.1)	1	100	94	100 (10)	1.00 (0.04)
		5	4.8 (2.0)	0	109	94	104 (5)	1.18 (0.04)
		10	4.8 (1.0)	1	87	91	**108 (7)**	**1.37 (0.18)**
		20	3.2 (1.9)	5	**64**	**84**	62* (19)	1.67* (0.23)
		40	1.6 (0.7)	11	33	57	22* (4)	3.01* (0.27)
Tunicamycin	4	**0**	4.4 (1.5)	2	100	95	100 (6)	1.00 (0.09)
		2	3.3 (1.5)	2	94	93	111 (6)	1.02 (0.04)
		4	1.9 (1.3)	3	93	93	104 (9)	0.89 (0.04)
		8	4.5 (2.8)	4	91	84	107 (8)	0.91 (0.02)
		20	2.7 (2.4)	3	**73**	**81**	**80 (16)**	**1.06 (0.06)**
	24	**0**	11.1 (3.3)	2	100	96	100 (3)	1.00 (0.04)
		2	1.6** (1.1)	0	59	88	44* (3)	3.65* (0.20)
		4	0.3** (0.2)	0	**49**	90	38* (3)	4.17* (0.33)
		8	0.3** (0.1)	0	42	80	32* (1)	4.5* (0.20)
		20	0.2** (0.1)	5	29	**70**	18* (5)	4.7* (1.00)

Bold values indicate the top concentrations selected for evaluation in the Comet assay based on each endpoint.

[a] DNA damage is represented by the percentage of DNA fragments present in the Comet tail (% tail intensity). Each point represents the mean of medians of three replicates and in parentheses the standard deviation.

[b] Percentage of 'clouds' is represented by the percentage of highly damaged cells without a discernable head but with a large diffuse tail. Each point represents the mean of three replicates at each concentration.

[c] Relative cell number is represented by the percentage of the mean of the cell numbers of three replicates at each concentration relative to the mean of the cell numbers of the three solvent control replicates.

[d] Absolute viability is represented by the percentage of viable cells against total cells determined by the trypan blue dye exclusion assay. Each point represents the mean of three replicates.

[e] Relative ATP activity is represented by the percentage of the mean of the ATP amount in three replicates at each concentration relative to the mean of the ATP amount in the three solvent control replicates and in parentheses the standard deviation.

[f] Relative Caspase-3/7 activation is represented by the fold change of the mean of the activated Caspase-3/7 in three replicates at each concentration relative to the mean of the activated Caspase-3/7 in the three solvent control replicates and in parentheses the standard deviation.

* One-way ANOVA with Dunnett's correction ($p < 0.05$); decreased ATP or increased Caspase-3/7 activation.

** One-way ANOVA with Dunnett's correction ($p < 0.05$); decreased % tail intensity.

Table 3
DNA damage, cytotoxicity and apoptosis induced by genotoxic chemicals.

Chemical	Treatment (h)	Concentration (μg/mL)	% Tail intensity [mean (SD)][a]	% Clouds[b]	Relative cell number (%)[c]	Absolute viability (%)[d]	Relative ATP activity (%) [mean (SD)][e]	Relative Caspase-3/7 activation [mean (SD)][f]
MMS	4	**0**	6.1 (2.2)	2	100	91	100 (5)	1.00 (0.01)
		5	34.5* (1.5)	2	97	93	102 (3)	0.96 (0.12)
		10	49.1* (11.5)	4	101	90	94 (5)	0.91 (0.15)
		20	65.6* (6.9)	2	97	93	105 (4)	0.97 (0.12)
		50	89.3* (0.9)	5	**93**	**91**	**105 (3)**	**0.90 (0.20)**
	24	**0**	11.5 (0.7)	4	100	90	100 (1)	1.00 (0.03)
		5	21.5* (2.9)	4	78	91	97 (3)	1.04 (0.03)
		10	42.0* (1.8)	2	70	92	96 (5)	**1.25 (0.08)**
		20	64.0* (3.8)	2	**59**	**89**	**93 (5)**	2.08* (0.05)
		50	93.0* (1.3)	90	39	48	45* (4)	9.93* (0.62)
EMS	4	**0**	4.1 (2.4)	3	100	83	100 (7)	1.00 (0.12)
		125	78.6* (5.1)	4	107	73	**97 (2)**	0.76 (0.03)
		250	87.0* (9.9)	38	110	84	79* (6)	0.84 (0.15)
		500	97.9* (1.4)	100	115	76	71* (9)	0.64 (0.02)
		1000	NA	100	**107**	**76**	74* (11)	**0.98 (0.13)**
	24	**0**	5.5 (5.9)	24	100	89	100 (2)	1.00 (0.03)
		7.8	10.8 (7.0)	16	92	89	102 (3)	**0.98 (0.02)**
		15.6	21.0 (6.3)	10	82	91	104 (3)	1.20* (0.02)
		31.25	54.6* (25.4)	67	82	92	**100 (5)**	1.77* (0.10)
		62.5	62.3* (6.6)	100	73	87	85* (8)	2.42* (0.10)
		125	94.9* (3.5)	100	61	88	80* (8)	3.07* (0.11)
		250	94.6* (4.2)	100	**49**	**83**	65* (2)	4.26* (0.09)
Etoposide	4	**0**	5.2 (2.3)	0	100	88	100 (3)	1.00 (0.38)
		1.25	80.0* (6.3)	4	81	85	109 (22)	1.03 (0.02)
		2.5	93.6* (0.1)	88	85	85	94 (4)	0.75 (0.10)
		5	95.8* (2.0)	94	76	75	107 (21)	0.70 (0.14)
		10	94.6* (4.5)	94	**72**	**82**	**112 (8)**	**0.63 (0.09)**
	24	**0**	9.8 (1.2)	0	100	88	100 (10)	1.00 (0.33)
		0.125	24.8* (5.0)	2	39	**69**	101 (20)	**1.07 (0.12)**
		0.25	43.2* (0.6)	3	33	49	94 (18)	2.61* (0.35)
		0.5	52.0* (1.6)	9	46	18	**77 (5)**	5.34* (0.07)
		1	59.5* (2.0)	16	29	17	43* (3)	6.19* (0.39)
4-NQO	4	**0**	4.4 (0.6)	1	100	75	100 (19)	1.00 (0.08)
		0.025	26.8* (5.7)	0	78	68	84 (24)	**1.06 (0.23)**
		0.05	39.2* (1.0)	0	83	77	104 (18)	1.57* (0.39)
		0.2	80.6* (4.2)	4	60	74	152** (29)	1.58* (0.21)
		0.5	93.2*	99	**63**	**71**	**132 (12)**	1.04 (0.13)
	24	**0**	3.9 (2.6)	10	100	91	100 (2)	1.00 (0.03)
		0.00313	3.4 (2.7)	15	87	88	101 (4)	0.99 (0.03)
		0.00625	10.7 (6.4)	34	93	87	**104 (2)**	**1.10 (0.02)**
		0.0125	19.4 (24.0)	37	78	85	81* (1)	1.69* (0.07)
		0.025	29.3 (26.0)	57	**59**	**69**	71* (6)	2.70* (0.21)
		0.05	42.7 (8.4)	45	39	38	35* (9)	4.72* (0.17)

NA, not available. Bold values indicate the top concentrations selected for evaluation in the Comet assay based on each endpoint.

[a] DNA damage is represented by the percentage of DNA fragments present in the Comet tail (% tail intensity). Each point represents the mean of medians of three replicates and in parentheses the standard deviation.

[b] Percentage of 'clouds' is represented by the percentage of highly damaged cells without a discernable head but with a large diffuse tail. Each point represents the mean of three replicates at each concentration.

[c] Relative cell number is represented by the percentage of the mean of the cell numbers of three replicates at each concentration relative to the mean of the cell numbers of the three solvent control replicates.

[d] Absolute viability is represented by the percentage of viable cells against total cells determined by the trypan blue dye exclusion assay. Each point represents the mean of three replicates.

[e] Relative ATP activity is represented by the percentage of the mean of the ATP amount in three replicates at each concentration relative to the mean of the ATP amount in the three solvent control replicates and in parentheses the standard deviation.

[f] Relative Caspase-3/7 activation is represented by the fold change of the mean of the activated Caspase-3/7 in three replicates at each concentration relative to the mean of the activated Caspase-3/7 in the three solvent control replicates and in parentheses the standard deviation.

* One-way ANOVA with Dunnett's correction ($p < 0.05$); increased % tail intensity, Caspase-3/7 activation or decreased ATP.

** One-way ANOVA with Dunnett's correction ($p < 0.05$), increased ATP.

in too few cells to score (data not shown). Therefore, cytotoxicity and DNA damage were evaluated at 2, 4, 8 and 20 μg/mL (Fig. 4 and Table 2). At 4 h, none of the endpoints showed significant cytotoxicity; therefore, 20 μg/mL should be selected as the top concentration in the Comet assay based on any of the three criteria (see Section 4 and Table 4). At 24 h, relative cell number was reduced to 49% at 4 μg/mL, and viability was reduced to 70% at 20 μg/mL; whereas the decrease in ATP and the induction of Caspase-3/7 activation were significant at all four concentrations (Fig. 4B and Table 2). Therefore, at 24 h, 20 μg/mL should be selected as the top concentration for the Comet assay based on viability, and 4 or <2 μg/mL should be chosen based on relative cell number or ATP/Caspase, respectively (see Section 4 and Table 4). The Comet assay results showed no increase of DNA damage at either time point; instead, tunicamycin induced a concentration-dependent decrease in % tail intensity at 24 h (Fig. 4D and Table 2), suggesting the potential presence of DNA- or protein-cross-linking effect. However, the decrease of % tail intensity was observed at 24 h but not at 4 h, which implies

Table 4
Top concentration determined by relative cell number, trypan blue exclusion (TB), biomarkers of cytotoxicity or high percentage of clouds.

Chemical	Treatment (h)	Top concentration (μg/mL) based on ≥50% relative cell number [DNA damage][a]	Top concentration (μg/mL) based on ≥70% TB [DNA damage][a]	Top concentration (μg/mL) based on ATP and Caspase-3/7 activation [DNA damage][a]	Top concentration (μg/mL) limited by >30% clouds [DNA damage][a]
Glucose	4	1800 [−]	450 [−]	1800 [−]	NA
	24	1800 [−]	1800 [−]	1800 [−]	NA
Ethanol	4	450 [−]	225 [−]	450 [−]	NA
	24	450 [−]	112.5 [−]	450 [−]	NA
2,4-DCP	4	40 [−]	20 [−]	10 [−]	NA
	24	20 [−]	20 [−]	10 [−]	NA
Tunicamycin	4	20[b] [−]	20[b] [−]	20[b] [−]	NA
	24	4 [−][#]	20 [−][#]	<2 [ND]	NA
MMS	4	50[b] [+]	50[b] [+]	50[b] [+]	NA
	24	20 [+]	20 [+]	10 [+]	20 [+]
EMS	4	>1000 [+]	>1000 [+]	125 [+]	125 [+]
	24	250 [+]	>250 [+]	7.8 [−]	15.6 [−]
Etoposide	4	>10 [+]	>10 [+]	>10 [+]	1.25 [+]
	24	<0.125 [ND]	0.125 [+]	0.125 [+]	NA
4-NQO	4	>0.5 [+]	0.5 [+]	0.025 [+]	0.2 [+]
	24	0.025 [−]	0.025 [−]	0.00625 [−]	0.00313 [−]

[−], negative; [+], positive; [−][#], possible cross-linker; ND, not determined based on the available data; NA, not applicable.
[a] DNA damage results from the Comet assay.
[b] Too few scorable cells at the next higher concentration.

that it may require longer than 4 h for tunicamycin to interact with DNA. Moreover, tunicamycin has not been reported with any DNA-interactive activity and it did not induce micronucleus formation in CHO-K1 cells [24], so this decrease in DNA migration remains to be further investigated.

Taken together, our data showed that these two chemicals induced significant cytotoxicity in the treated cells, but did not cause an increase in DNA migration in the Comet assay. Tunicamycin was expected to be a non-genotoxic chemical, but our results showed that it is possibly a DNA- or protein-cross-linker. The maximum concentrations selected by ATP/Caspase endpoints were lower than those determined by trypan blue assay or relative cell number.

3.3. Genotoxic and cytotoxic chemicals: MMS, EMS, etoposide and 4-NQO

MMS was tested up to 1100 μg/mL (10 mM); however, only the data from concentrations up to 50 μg/mL are presented and discussed here, because all the treated cells were unscorable (all clouds) at concentrations of 50 μg/mL and above (Fig. 5C, D and Table 3). At 4 h, over this concentration range (5–50 μg/mL), with no cytotoxicity detected by any endpoint, a statistically significant and concentration-dependent increase in DNA damage was observed in MMS-treated cells compared with the solvent control ($p < 0.05$) (Fig. 5C and Table 3). At 24 h, MMS treatment resulted in a significant decrease in the relative cell number, viability and ATP at the

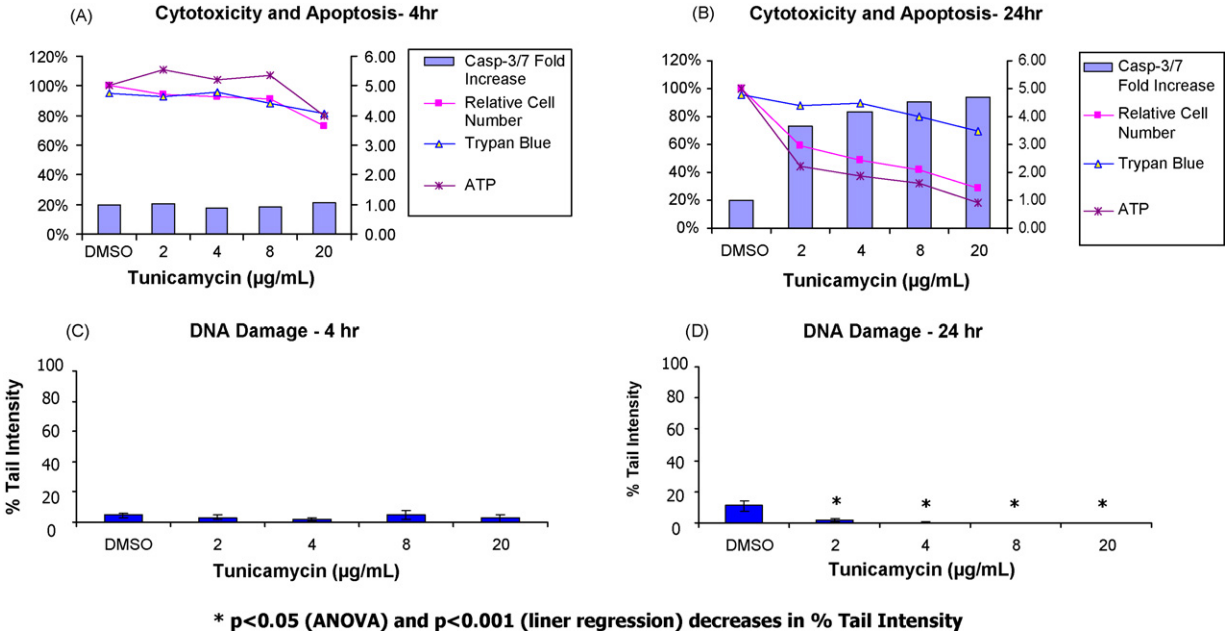

Fig. 4. Cytotoxicity and DNA damage induced by tunicamycin treatment. TK6 cells were treated with tunicamycin for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

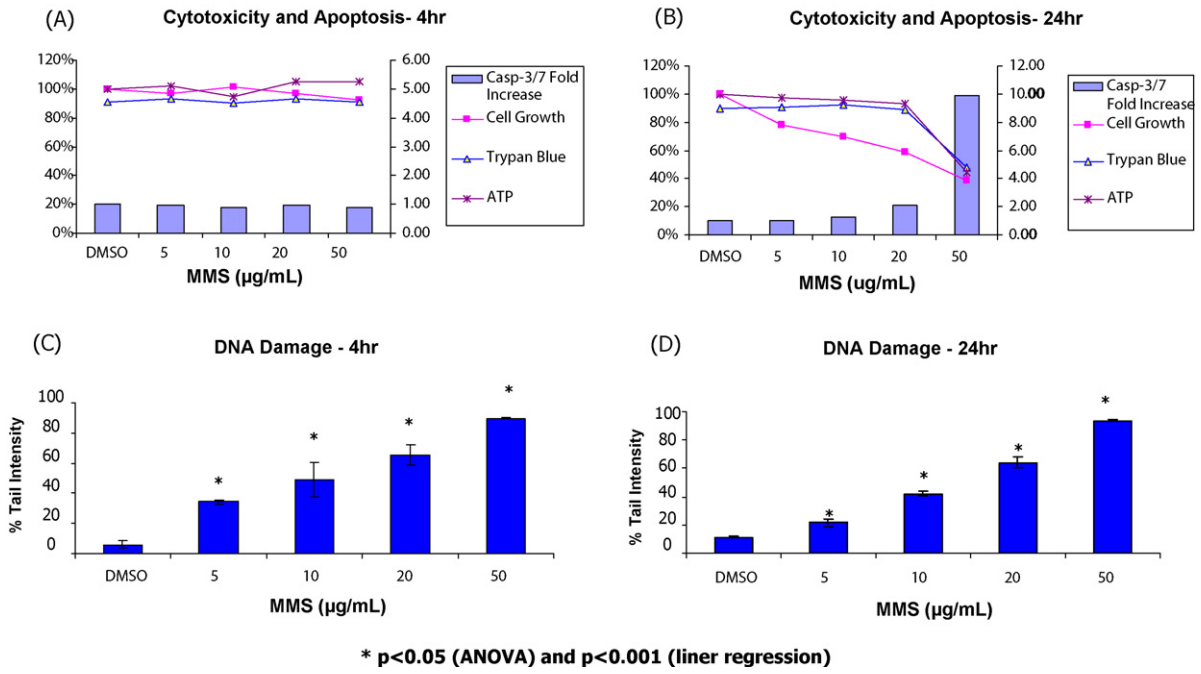

Fig. 5. Cytotoxicity and DNA damage induced by MMS treatment. TK6 cells were treated with MMS for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

highest concentration only, while Caspase-3/7 activation was seen for the highest two concentrations (Fig. 5B and Table 3). Therefore, 20 μg/mL should be the highest concentration to be evaluated in the Comet assay at 24 h based on relative cell number or viability; and 10 μg/mL should be selected based on ATP/Caspase responses (see Section 4 and Table 4). In addition, MMS at concentrations above 20 μg/mL also displayed a high percentage of 'clouds', another indication of cytotoxicity (Table 3). Similar to the Comet results from the 4-h treatment, a statistically significant increase in DNA damage was observed in MMS-treated cells in a concentration-dependent manner ($p < 0.05$).

EMS was examined up to 1000 μg/mL, which was selected based on Henderson et al. [22] (Fig. 6 and Table 3). EMS treatment for 4 h induced a slight but significant reduction of ATP levels at 250 μg/mL and above, but had little effect on cell number, viability and Caspase-3/7 activation (Fig. 6A and Table 3). Concentrations of 250 μg/mL and above produced a high percentage of clouds (>30%) and therefore should be excluded from evaluation of DNA damage in the Comet assay. Thus, the top concentration, 125 μg/mL, selected by the ATP/Caspase endpoints for the 4-h treatment were in agreement with that limited by the high percentage of 'clouds' (Table 4). Data from the Comet assay showed a dose-dependent increase in DNA damage from 125 to 500 μg/mL ($p < 0.05$), confirming the genotoxicity of this chemical. At 24 h, treatment with EMS reduced relative cell number to 49% only at the highest concentration whereas viability was essentially unchanged. The decrease of ATP levels and activation of Caspase-3/7 were significant starting from 62.5 and 15.6 μg/mL, respectively (Fig. 6B and Table 3). Therefore, 250 μg/mL or above should be selected as the top concentration for the Comet assay based on relative cell number or viability, respectively; whereas 7.8 μg/mL should be chosen according to ATP/Caspase responses (see Section 4 and Table 4). Concentrations of 31.25 μg/mL and above showed a high percentage of 'clouds' (>30%) (Table 3), which allows only the lowest two concentrations being evaluated in the Comet assay. The increases in DNA damage were statistically significant only at concentrations of 31.25 μg/mL and above (Fig. 6D and Table 3) at 24 h, suggesting the 24-h treatment is probably not appropriate to separate the genotoxic and cytotoxic effects.

Etoposide was tested up to 10 μg/mL [26] (Fig. 7). At 4 h, none of the endpoints showed substantial cytotoxicity and no Caspase activation was observed. However, a high percentage of 'clouds' (>30%) was observed at 2.5, 5 and 10 μg/mL (Fig. 7A and Table 3). Therefore, the top concentration selected in the Comet assay should be 1.25 μg/mL at 4 h. As a result, the increase of DNA damage at 1.25 μg/mL was statistically significant, which appropriately predicted the genotoxicity of this chemical. In addition, an independent experiment which tested etoposide in the Comet assay at 0.125, 0.25, 0.5 and 1 μg/mL showed a concentration-dependent increase of DNA damage (data not shown). At 24 h, relative cell number at all four concentrations was below 50%. Trypan blue dye exclusion assay and Caspase-3/7 activation showed significant cytotoxicity at concentrations of 0.25 μg/mL and above while reduction of ATP was significant only at 1 μg/mL (Fig. 7B and Table 3). The top concentration to be selected in the Comet assay should be 0.125 μg/mL based on viability or ATP/Caspase, or <0.125 μg/mL based on relative cell number (see Section 4 and Table 4). There were statistically significant increases in DNA damage at 0.125 μg/mL at 24 h (Fig. 7D and Table 3). We did not test etoposide at concentrations below 0.125 μg/mL, but it is reasonable to argue that it may not induce significant DNA damage at these low doses as the % tail intensity resulted from etoposide treatment at 0.125 μg/mL was only around 2-fold compared with the solvent control. Therefore, the top concentration selected for the Comet assay based on relative cell number may be inappropriate for etoposide.

4-NQO was examined up to 0.5 μg/mL [22] (Fig. 8). At 4 h, the highest concentration (0.5 μg/mL) reduced viability to 71%, but did not decrease relative cell number to below 50% (Fig. 8A and Table 4). ATP levels increased drastically at the two top concentrations (significant at 0.2 μg/mL), suggesting a short-term stimulation of ATP generation in response to the treatment. Caspase-3/7 was activated modestly but significantly at 0.1 and 0.2 μg/mL but not at 0.5 μg/mL, possibly due to decreased cell number and viability at

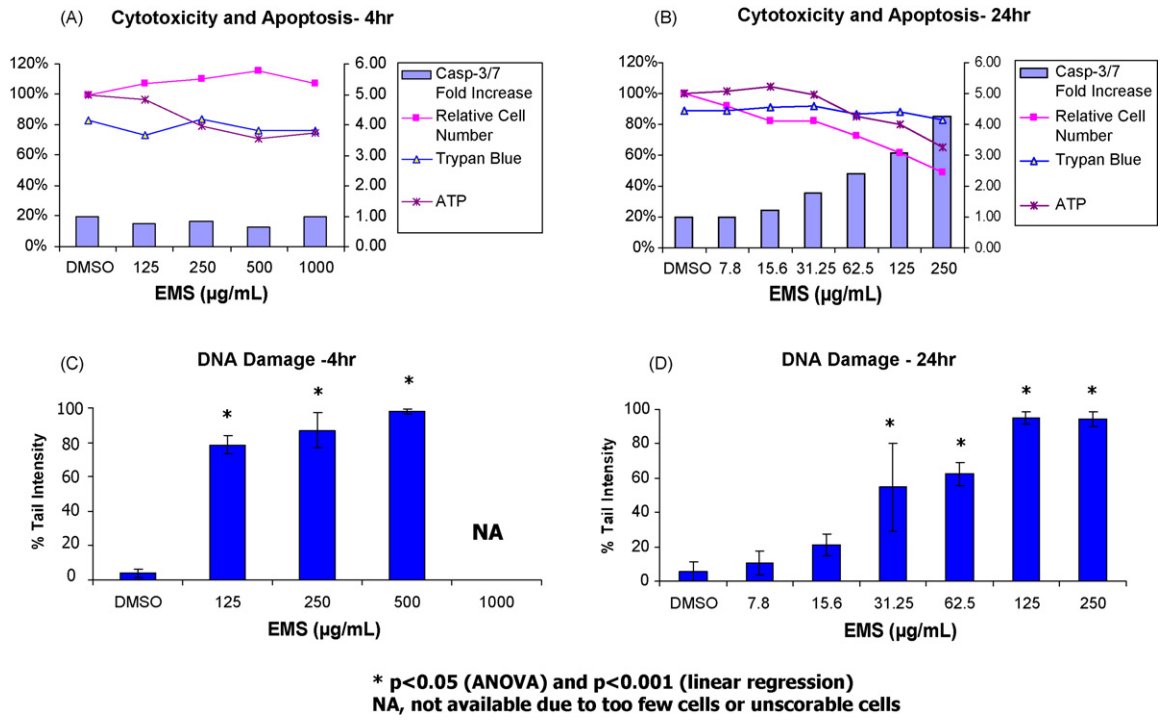

Fig. 6. Cytotoxicity and DNA damage induced by EMS treatment. TK6 cells were treated with EMS for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

this concentration. Thus, a top concentration of 0.5 μg/mL should be selected based on viability or >0.5 μg/mL based on relative cell number; or 0.05 μg/mL based on ATP/Caspase for the Comet assay (see Section 4 and Table 4). However, 0.5 μg/mL should be excluded from the Comet assay due to the high percentage of 'clouds' (Table 3). The increases of DNA damage were statistically significant at all four concentrations ($p < 0.05$) and the increase was concentration-dependent (Fig. 8C and Table 3), suggesting a correct conclusion of genotoxicity based on all three criteria for this chemical. At 24 h, 4-NQO treatment resulted in significant decreases in relative cell number (<50%) and viability ($\lesssim$70%) only at the highest concentration. The reduction of ATP and activation

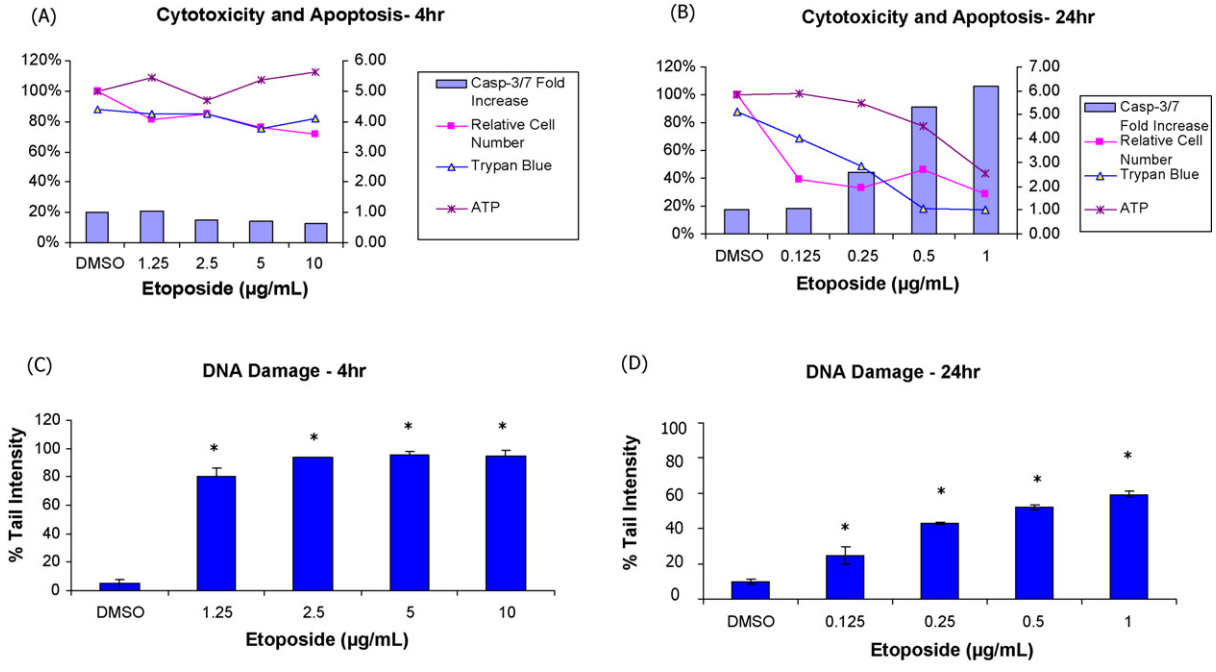

Fig. 7. Cytotoxicity and DNA damage induced by etoposide treatment. TK6 cells were treated with etoposide for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

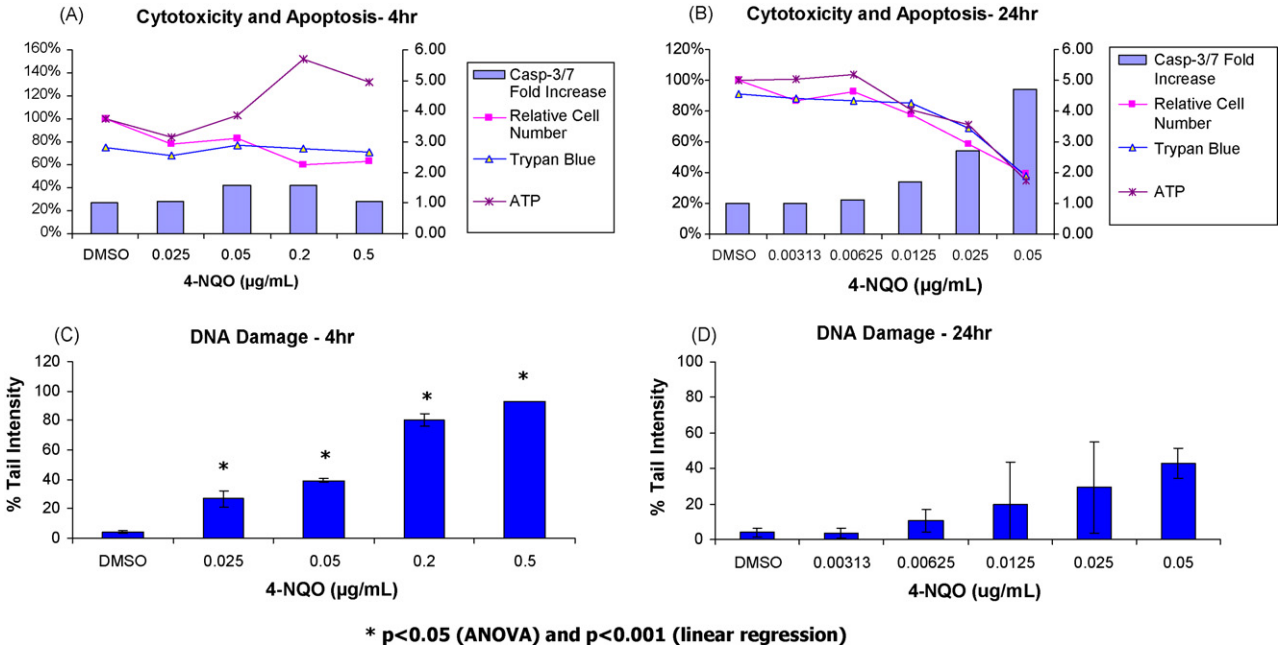

Fig. 8. Cytotoxicity and DNA damage induced by 4-NQO treatment. TK6 cells were treated with 4-NQO for 4 h (A and C) or 24 h (B and D). Various cytotoxicity (relative cell number, trypan blue and ATP) and apoptosis endpoints (Caspase-3/7 activation) were determined at each time point (A and B). DNA damage in the treated and control samples was examined by the Comet assay and represented by % tail intensity (C and D).

of Caspase-3/7 were significant at concentrations of 0.0125 μg/mL and above (Fig. 8B and Table 3). The top concentration selected for the Comet assay should be 0.025 μg/mL based on relative cell number or viability, or 0.00625 μg/mL based on ATP/Caspase responses (see Section 4 and Table 4). Notably, there was a high percentage of 'clouds' (>30%) at 0.00625 μg/mL and above, so the top concentration based on this criterion will be 0.00313 μg/mL (Table 3). As a result, in the Comet assay, although the mean % tail intensity was increased in a dose-dependent manner in cells treated with 4-NQO at concentrations of 0.00625 μg/mL and above, the differences were not statistically significant, possibly due to the heterogeneous response at such low concentrations.

In agreement with the findings with non-genotoxic but cytotoxic chemicals, the top concentrations selected based on ATP/Caspase were generally lower than that based on trypan blue or relative cell number except for etoposide at 24 h (Table 4), and more close to the concentrations limited by high percentage of 'clouds'. The 4-h treatment correctly predicted all four genotoxic, cytotoxic chemicals and appeared to be the better time point to conduct the *in vitro* Comet assay.

4. Discussion

Cytotoxicity assessment is a crucial element in *in vitro* genotoxicity testing. The determination of high toxic concentrations and testing below those concentrations allow an appropriate detection of genotoxic damage, in this case DNA strand breaks (Comet assay). In the Comet assay and other genotoxic assays, excessive cytotoxicity can lead to irrelevant positive results. Cytotoxicity endpoints have been used in *in vitro* genetic toxicology tests for decades, but finding assays that permit a fast and reliable way of measuring cytotoxicity, is currently being revisited [27,28].

Here, we compared four cytotoxicity endpoints in the *in vitro* Comet assay using TK6 cells while testing three representative classes of chemicals based on cytotoxicity and genotoxicity. The trypan blue dye exclusion assay has been used routinely as the only cytotoxicity measurement in the *in vitro* Comet assay. Henderson et al. [22] examined a number of cytotoxins along with genotoxins in TK6 cells and found that two cytotoxins (sodium lauryl sulphate and potassium cyanide) showed significant increase in DNA migration when cell survival (viability) was lower than 70%, as determined by trypan blue dye exclusion assay. The authors suggested only scoring Comet slides with ≥70% viability in order to avoid false positive results due to cytotoxicity. This recommendation was followed by many laboratories conducting the Comet assay and it provided a relatively effective guideline to minimize false positive findings associated with cytotoxicity. However, since trypan blue dye exclusion assay is based on compromised cell membrane integrity which frequently occurs only at late stages of apoptosis or necrosis, it is reasonable to argue that it often underestimates cytotoxicity. Our data in the current study demonstrated relatively lower sensitivity of trypan blue dye exclusion assay compared to other cytotoxicity methods (Figs. 4B and 6B). Moreover, as loss of membrane integrity could be impacted by several other factors, such as mechanical damage and osmolarity, the trypan blue assay tends to produce unanticipated response when used to measure cytotoxicity for cells exposed to nonhazardous (non-genotoxic and non-cytotoxic) chemicals at high concentrations (Figs. 1A and 2). Some laboratories determine relative cell number (or cell counts) along with the trypan blue dye exclusion assay. The resulting relative cell survival, a product of total cell number and viability, usually provides a better estimation of cytotoxicity. However, measurement of both cell counts and viability is a labor-intensive and time-consuming effort using systems that are not automated.

In this study, we explored the possibility of using biomarker-based cytotoxicity and apoptosis assays for dose selection in *in vitro* Comet assay. The ATP bioluminescent assay directly determines the metabolic status of a cell population and/or the number of cells in a culture, which provides a sensitive, robust and consistent measurement of cytotoxicity. The Caspase-3/7 activation assay detects mid- to late-stage apoptosis, which represents a cellular state of excessive toxicity. As increased DNA damage can be a result of cytotoxicity instead of genotoxicity, the Caspase-3/7 activation assay can serve as a sensitive biomarker to monitor apoptosis-related cytotoxicity. There are limitations of using ATP

quantification or Caspase-3/7 assays as cytotoxicity measurement alone. It should be noted that the cellular ATP levels were elevated when treated by some chemicals at relatively low (non-lethal) concentrations (Figs. 3A, 5B and 8A) as chemical treatment resulted in more reduction on cell number than on levels of ATP at several tested concentrations, which yielded elevated ATP levels per cell in these treated samples compared to the solvent control. The transient increase of the cellular ATP content in response to chemical treatment has been reported previously in bacteria and eukaryotic cancer cells and is possibly due to enhanced energy production when cells are undergoing self-repair in response to low concentration of toxins shortly after exposure [29–32]. Moreover, since, in eukaryotic organisms, ATP is mainly produced in the process of cellular respiration which takes place in the mitochondria and cytosol, chemicals that affect this process may impair the accuracy of using ATP amount as a cytotoxic measurement. On the other hand, the Caspase-3/7 activation assay cannot work with chemicals that directly inhibit Caspase activation, such as tetramethylthiuram [33], and it does not detect non-apoptotic cell death. However, combination of these two assays measures both metabolic and apoptotic status of the target cells, and compensates the limitations of each individual assay, and therefore can provide sensitive and reliable estimation of cytotoxicity.

We compared the data from the commonly used cytotoxicity endpoint – trypan blue dye exclusion assay with the results from the other three cytotoxicity measurements – relative cell number, ATP and Caspase-3/7 activation. Since ~50% of cytotoxicity (measured by relative cell number, population doubling, mitotic index or other calculations based on cell counts) is being used for top concentration selection in CA or MN, here we tentatively use 50% relative cell number as a criterion. The selected highest concentrations for the tested chemicals based on viability $\gtrsim$70%, relative cell number $\gtrsim$50%, or ATP/Caspase-3/7 activation (statistical analysis) are listed in Table 4. The majority of the selected concentrations from the ATP/Caspase-based criteria were equal to or lower than that based on the viability or relative cell number results, suggesting a more stringent selection using the biomarkers to cut off cytotoxic concentrations which may interfere with genotoxicity testing. The validation matrix of chemicals tested here for genotoxicity by the Comet assay appropriately predicted their positive or negative genotoxic potential using any of these criteria following a 4-h treatment. However, it is very likely the choice of cytotoxicity endpoint will make a difference in testing chemicals with "steep" toxicity curves and small concentration ranges to distinguish cytotoxic and genotoxic effects. Careful evaluation of a larger number of well-characterized chemicals is required to set the dose selection criteria. Statistical analysis of the ATP and the Caspase-3/7 activation data showed that when ATP was less than 80% of control or Caspase-3/7 activation was greater than 1.5-fold, the differences between the treated samples and control were generally significant. To simplify dose selection based on cytotoxicity, we suggest an initial standard of only scoring slides with relative ATP $\geq$80% and fold change of Caspase-3/7 activation <1.5 be used in the *in vitro* Comet assay in TK6 cells.

In addition to the investigation of cytotoxicity methods, we also evaluated the 4-h and the 24-h treatment/sampling time in the *in vitro* Comet assay. All tested chemicals were correctly judged positive or negative in genotoxicity based on DNA damage at 4 h, but 4-NQO was incorrectly predicted negative at 24 h. Also, EMS treatment resulted in insignificant increases in % tail intensity at low, non-cytotoxic concentrations at 24 h. The 24-h treatment generally induced more toxicity of the cells and resulted in lower maximum concentrations to be evaluated in the Comet assay compared with the 4-h treatment. Also, the ability of the cells to repair low levels of DNA damage and the loss of highly damaged cells could adversely affect the sensitivity of the Comet assay. On the other hand, the 24-h treatment could potentially capture DNA damage that resulted from some slow-acting chemicals or chemicals that require relatively longer period of time to be metabolized into DNA-reactive intermediates and substrates, which cannot be achieved by the 4-h treatment. We did not test chemicals that require metabolic activation in this study, because the primary objective of this work is to evaluate suitable cytotoxicity measurements. From the data here, the 4-h treatment/sampling time appears to be more appropriate to be used in the *in vitro* Comet assay, but whether the 24-h treatment/sampling time (4-h treatment plus 20-h recovery) provides advantages over 4-h treatment/sampling time with chemicals requiring metabolic activation remains to be examined.

Notably, a high percentage of 'clouds' has been regarded as an indication of excessive cytotoxicity or genotoxicity, so it also serves as a parameter to measure cytotoxicity [34]. Although there has not been a standard which defines test concentrations that produced a certain percentage of 'clouds' should be excluded from the DNA damage evaluation in the Comet assay, we tentatively excluded concentrations with >30% 'clouds'. The top concentrations for the Comet assay limited by 'clouds' are also listed in Table 4. Although whether formation of 'clouds' is an indication of apoptosis is still under discussion, our results suggested that the 'clouds' are unlikely to be the DNA fragments resulting from apoptosis because DNA fragmentation represents almost at the final stage of apoptosis [35] and it should occur no earlier than Caspase activation. For example, the 4-h treatment by EMS led to 100% of 'clouds' at 500 and 1000 μg/mL but no significant activation of Caspase-3/7 (Table 3 and Fig. 6A). Following 24 h of continuous treatment by tunicamycin, robust Caspase-3/7 activation and significant cytotoxicity were induced at all concentrations tested, but no significant 'clouds' were observed (Table 2 and Fig. 4B). From our point of view, the 'clouds' do represent excessive DNA damage and/or high cytotoxicity, but may not correlate well with apoptosis.

In conclusion, the data support the use of ATP and Caspase assays as cytotoxicity endpoints to be used to determine maximum concentrations in *in vitro* Comet assay in TK6 cells, and have great potentials to be applied in other cell lines and other *in vitro* genotoxicity assays.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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