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Comparative investigations of genotoxic activity of five nitriles in the comet assay and the Ames test

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ABSTRACT

Two short-term assays, the modified Ames test and the comet assay, were carried out to evaluate the genotoxicity of five nitriles (acetonitrile, propionitrile, methacrylonitrile, butyronitrile, and benzonitrile). With the comet assay, all the nitriles studied were found to induce the genotoxicity in human lymphocytes and Hep G2 cells. Except for butyronitrile, the genotoxic potency in lymphocytes was more pronounced than that in Hep G2 cells, and the rank order of genotoxicity induced by these five nitriles in lymphocytes was different from that in Hep G2 cells, indicating that the pathways leading to genotoxicity in both types of cells were different. In the modified Ames test, no tested nitriles showed mutagenic activity on *Salmonella typhimurium* strain TA 98 and TA 100 with and without metabolic activation.

Comparing the results obtained from both tests in this study, the comet assay seems to be more sensitive than the modified Ames test. Thus, the comet assay can be used to detect the genotoxicity of all nitriles.

1. Introduction

Aliphatic nitriles (R–CN), one group of compounds exhibiting the versatile physical and chemical properties, are used as the important solvent and intermediates in polymers, plastic, synthetic fibers, resins, dyestuffs, pharmaceuticals and vitamin industries [1]. The route of occupational exposure to aliphatic nitriles is via inhalation of vapors or aerosols and/or by skin absorption [1]. Some aliphatic nitriles such as butyronitrile, acetonitrile, and propionitrile were proved to exert their toxicity through cyanide liberation, leading to the inhibition of hepatic and brain cytochrome *c* oxidase in rat [1], and methacrylonitrile-related non-neoplastic lesions were seen in the nose and livers of rats [2]. In addition, animal studies have indicated additional effects of nitriles poisoning, including the development of duodenal ulcers, nuclear changes in neurons and satellite spinal ganglia, thyroid hyperemia and hyperplasia, neurogenic bladder dysfunction and adrenal apoplexy [3–5].

The genotoxicity of some tested nitriles including benzonitrile, acetonitrile, and methacrylonitrile studied in this study had been reported. Although the genotoxicity of acetonitrile has been investigated in numerous studies in mammalian and sub-mammalian test systems [6], these genotoxic results were inconsistent. Acetonitrile showed no evidence of point mutations in bacteria [7–9], cultured mammalian cells [10], and no point mutation or recombination *Saccharomyces cerevisiae* [11]. Additionally, acetonitrile was inactive in rat hepatocyte unscheduled DNA synthesis assays, both in *vitro and in vivo* [12]. In contrast, there are data to suggest that acetonitrile may induce aneuploidy in sub-mammalian test systems such as *S. cerevisiae* [11] and *Drosophila melanogaster* [13], whereas acetonitrile was proved to not to be either clastogenic or aneugenic in the bone marrow of the mouse at the maximum tolerated dose [6]. Methacrylonitrile showed non-genotoxicity both in a battery of short-term *in vitro* and *in vivo* test [2]. There was the limited data addressing the genotoxicity of benzonitrile, only in one study suggesting that benzonitrile can induce chromosomal genotoxicity in V79 cells [14].

The inconsistent or little information for genotoxicity of these five nitriles (acetonitrile, propionitrile, methacrylonitrile, butyronitrile and benzonitrile) which are one of the thermal decomposition products of a polyacrylonitrile yarn [15] prompted us to quantitatively assess, for the first time, the extent of these nitriles-induced DNA damage in a single-cell system, comet assay, and to ascertain their genotoxic and mutagenic activities. This systematic study has demonstrated the differential genotoxic potential of these tested compounds using specific and sensitive assay employing different cell types.

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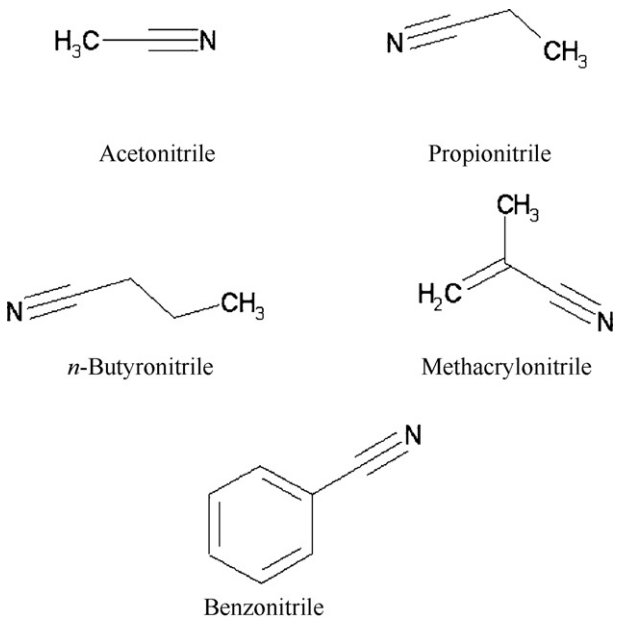

Fig. 1. Chemical structures of five nitriles.

2. Materials and methods

2.1. Chemicals

L-Glutamine, phosphate buffered saline (PBS; Ca^{2+}, Mg^{2+} free), RPMI 1640 medium, DMEM medium, and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (HyClone, USA). All five nitriles were obtained from Sigma–Aldrich Co. (St. Louis, MO), and were of analytical reagent grade. The structures of these nitriles were depicted in Fig. 1.

2.2. Isolation and treatment of lymphocytes for comet assay

Lymphocytes cultures were performed according to the procedures of Feng et al. [16]. Blood withdrawn from a female donor (health and non-smoker, aged 25) was collected into Ficoll-Hypague. The samples were then centrifuged at 200 × *g* at 25 °C for 20 min. The formed lymphocyte forming a layer was directly above the Ficoll-Hypague, The isolated lymphocytes (0.3 ml) were cultured in 4.7 ml RPMI 1640 medium including 20% heat-inactivated fetal calf serum, 2% phytohemagglutin (PHA), 100 IU/ml of penicillin, 100 μg of streptomycin, and 2 mM of L-glutamine at 37 °C under 5% CO_2 atmosphere. The cells were diluted down to a concentration of 2.5×10^5 cells/ml prior to use. Lymphocytes were incubated with different concentrations of the tested chemicals (50, 100, and 250, and 500 μM) dissolved in DMSO (1% as a final concentration) at 37 °C for 1 h in a dark incubator. Subsequently, the cells were centrifuged at 200 × *g* for 3 min at 4 °C and then were mixed with low melting point agar for the comet assay as described below.

2.3. Treatment of Hep G2 cells for comet assay

Hep G2 cells were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 50 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were grown in 25-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2. Subsequently, the cells were treated with different concentration of five nitriles (50, 100, 200 and 400 μM) dissolved in DMSO for 1 h in a dark incubator. Finally, the cells were collected by centrifugation (250 × *g* for 5 min at 4 °C. A single-cell suspension of 3×10^5 cells/ml was prepared in DMEM medium without any supplement for comet assay.

2.4. Cell viability analysis

The procedures were conducted following the procedures in Chen et al. [17]. A volume of 0.49 ml cell suspension treated with each tested chemical at the doses ranging from 0 μM to 200 μM was mixed with 10 μl of 0.4% trypan blue solution. Its viability was determined after 5 min of reaction. The cells were analyzed through microscopic observation to determine the percentage of viable cells.

2.5. Comet assay

The comet assay was performed under alkaline conditions following the method of our previous study [17]. Conventional microscope slides were dipped with a solution of 85 μl 0.5% of normal melting point agarose (NMPA) and 0.5% low melting point agarose (LMPA) in PBS (pH 7.4), and allowed to dry on a flat surface at room temperature. Ten microliters of cell suspension (2.5×10^5 cells/ml) was gently mixed with 75 μl of 0.5% (w/v) of LMPA in PBS (pH 7.4). Seventy-five microliter of this suspension was rapidly layered onto the slides pre-coated with the mixtures of 0.5% NMPA and 0.5% LMPA, and covered with a cover glass. The slides were maintained at 4 °C for 5 min, the cover glass was removed, and cells were immersed in a freshly made lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% (v/v) Triton X-100 at pH 10) at 4 °C for 1 h. The slides were then placed in a double row in a 260 mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA for 10 min. Thereafter, the electrophoresis (1 V/cm, 300 mA) was conducted for 15 min at 4 °C. After the electrophoresis, the slides were then soaked in a cold neutralizing buffer (400 mM Tris buffer, pH 7.5) at 4 °C for 10 min. Slides were dried in 100% methanol for 5 min, and stored in a low humidity environment before staining with 40 μl PI (2.5 μg/ml).

2.6. Quantification of the comet assay

The quantification of the comet assay was adopted with the visual scoring as in our recently published paper [18]. One hundreds comets on each slide were scored visually according to the relative intensity of the tail. An intensity score from class 0 (undamaged) to class 4 (severely damaged) (Fig. 2) was assigned to each cell. The final magnification was 400× in a fluorescence microscope. Thus, the total score for the 100 comets could range from 0 to 400 because the 100 cells were observed individually in each comet assay. The extent of DNA damage was analyzed and then scored by the same experienced person, using a specific pattern when moving along the slide. The method of the observation was barred in a blind way during which the observer had no knowledge of the identity of the slide.

2.7. Mutagenicity assay

Mutagenicity was assessed by the preincubation assay as described by Maron and Ames [19]. Briefly, 0.1 ml of overnight-grown ($1–2 \times 10^8$ cfu/ml) of both strains were treated separately for 30 min at 37 °C [20] with 250, 500, 1000, 1500, and 3000 μg/plate of five nitriles, both in the absence and the presence of a rat liver homogenate (S9). All the compounds were dissolved in dimethyl sulfoxide (DMSO), which was used at a final concentration of less than 1% (v/v).

For the mutagenicity assay, the controls and nitriles-treated cells with mixed with 3 ml of sterile top agar (0.6% agar and 0.5% NaCl containing 0.5 mM histidine and 0.5 mM biotin) and poured onto

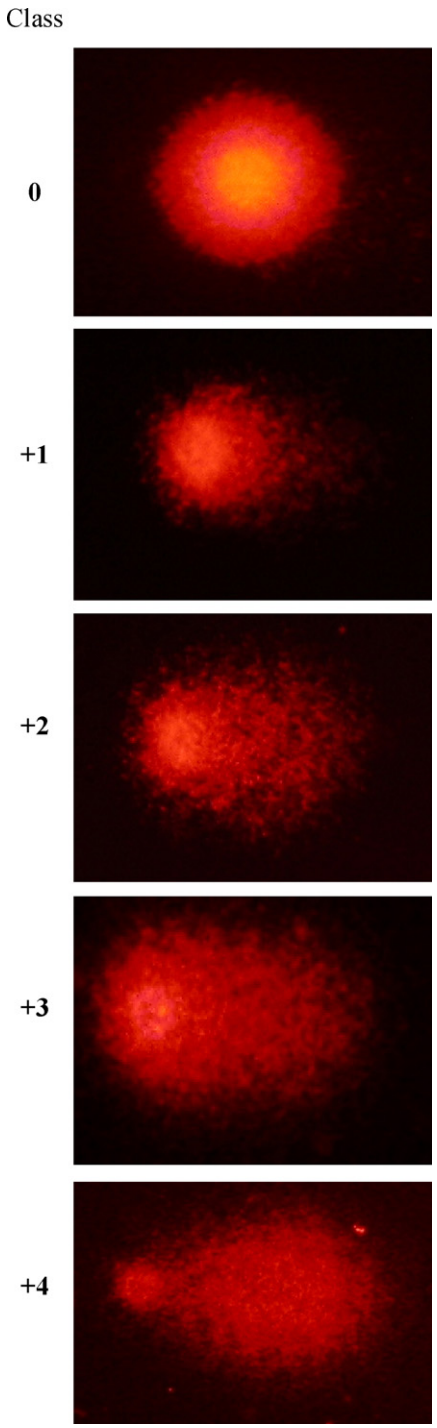

Fig. 2. Comet images illustrating the visual scoring classification (0–4).

minimal glucose agar plate [1× Vogel–Blonner salts (0.2 g/l magnesium sulfate, 2 g/l citric acid monohydrate, 10 g/l dipotassium hydrogen phosphate, and 3.5 g/l sodium ammonium phosphate), 2% glucose, and 1.5% agar]. The plates were then incubated at 37 °C for 48 h. Revertant colonies appeared on a background lawn of bacteria. Two independent experiments were conducted; each experiment consisted of three replicate plates for each treatment.

2.8. Statistics

Images from 300 random cells (100 from triplicate slide) were analyzed for each experiment. The experiment (and not the cell) was used as the experiment unit. We followed the statistical method of Bajpayee et al. [20]. The homogeneity of variance between treatment groups was ascertained prior to the statistical analysis of the comet assay data. The mean comet data (DNA damage scores) were analyzed using one-way analysis of variance (ANOVA), with DNA damage as the dependant variable and concentrations of the tested compounds as the independent variable. If a significant *F*-value was obtained, then Dunnett's multiple comparison tests were conducted. $P < 0.05$ was considered to be significant.

For the mutagenicity assessment, induced responses were considered marginally positive when they were > or = two-fold background colonies and positive when they were > or = three-fold background colonies.

3. Results

3.1. DNA damage

The viability of lymphocytes cells and Hep G2 cells treated with five nitriles at the indicated doses for 1 h was evaluated. It was observed that the cell viability after the treatment of these compounds below 500 μM or 400 μM was at least >95% using trypan blue dye assays (data not shown). Thus, five nitriles at the maximum sub-lethal doses below 500 μM or 400 μM were used separately for the determination of genotoxicity in lymphocytes or Hep G2 cells using the comet assay. Table 1 summarized the results of DNA damage in human lymphocytes treated with varying concentrations of tested nitriles at 37 °C for 1 h, as measure by the alkaline comet assay. Results indicated that the positive group (cells

Table 1
The DNA damage in lymphocytes treated with different doses for 1 h. H_2O_2 was used as the positive control.

Chemicals	Dose (μM)	DNA Damage (mean ± S.D., arbitrary units)[a]
Acetonitrile	0	48 ± 9
	50	98 ± 24[b]
	100	190 ± 34[b]
	250	266 ± 46[b]
	500	221 ± 77[b]
H_2O_2	100	252 ± 50[b]
Propionitrile	0	57 ± 12
	50	66 ± 37
	100	107 ± 60[b]
	250	214 ± 40[b]
	500	193 ± 45[b]
H_2O_2	100	243 ± 31[b]
Methacrylonitrile	0	61 ± 5
	50	19 ± 5
	100	26 ± 9
	250	93 ± 12[b]
	500	150 ± 27[b]
H_2O_2	100	270 ± 44[b]
Butyronitrile	0	71 ± 27
	50	138 ± 55[b]
	100	149 ± 27[b]
	250	192 ± 35[b]
	500	210 ± 50[b]
H_2O_2	100	257 ± 63[b]
Benzonitrile	0	54 ± 22
	50	73 ± 1
	100	121 ± 8[b]
	250	169 ± 21[b]
	500	144 ± 12[b]
H_2O_2	100	157 ± 11[b]

[a] The scores of DNA damage were calculated from the respective values of at least three treatments (100 cells/slide, duplicates slides/treatment).
[b] $p < 0.01$.

Table 2
DNA damage in HepG2 cells treated with different doses for 1 h. H_2O_2 was used as the positive control.

Chemicals	Dose (μM)	DNA Damage (mean ± S.D., arbitrary units)[a]
Acetonitrile	0	38 ± 3
	50	37 ± 2
	100	83 ± 5[b]
	200	105 ± 22[b]
	400	215 ± 23[b]
H_2O_2	100	165 ± 54[b]
Propionitrile	0	29 ± 2
	50	37 ± 7
	100	56 ± 3[b]
	200	63 ± 9[b]
	400	81 ± 14[b]
H_2O_2	100	103 ± 17[b]
Methacrylonitrile	0	33 ± 10
	50	35 ± 7
	100	50 ± 12
	200	61 ± 6[b]
	400	68 ± 12[b]
H_2O_2	100	81 ± 13[b]
Butyronitrile	0	20 ± 2
	50	24 ± 5
	100	40 ± 10[b]
	200	153 ± 53[b]
	400	162 ± 24[b]
H2O2	100	152 ± 38[b]
Benzonitrile	0	65 ± 13
	50	68 ± 9
	100	77 ± 11
	200	80 ± 8
	400	88 ± 18[b]
H_2O_2	100	136 ± 13[b]

[a] The scores of DNA damage were calculated from the respective values of at least three treatments (100 cells/slide, duplicates slides/treatment).
[b] $p < 0.05$.

pretreated with 100 μM H_2O_2) showed significant levels of DNA damage score, while the negative control (0.5% DMSO as solvent for these tested nitriles) revealed very low DNA damage score. At a concentration of 50 μM, acetonitrile and butyronitrile exhibited significant DNA damage when compared to the negative control group ($p < 0.05$), while all five tested nitriles revealed the genotoxicity to lymphocytes at 250 μM. In the Hep G2 cells (Table 2), except for benzonitrile, other four nitriles at the dose of 200 μM showed the genotoxicity to cells, as compared to the control group (0.5% DMSO) ($p < 0.05$). This result indicated that these nitriles are DNA-damaging chemicals.

All the tested nitriles produced a significant concentration-dependent DNA damage (($p < 0.05$) in human lymphocyte and Hep G2 cells as measured by the comet assay (Table 3). Using the analysis of linear regression for the genotoxicity potency induced by these nitriles, we found that acetonitrile produced the greatest amount of damage in lymphocytes and Hep G2 cells, while butyronitrile and methacrylonitrile was the least DNA-damaging chemical in the lymphocytes and Hep G2 cells, respectively.

Table 3
Comparison of potency of DNA damage between lymphocytes and Hep G2 cells treated with the tested nitriles.

Chemicals	Potency of DNA damage (arbitrary units/μM)[a]	
	Lymphocytes	Hep G2 cells
Acetonitrile	0.86 (0.96)[b]	0.48 (0.99)
Propionitrile	0.66 (0.99)	0.13 (0.96)
Methacrylonitrile	0.30 (0.99)	0.091(0.93)
Butyronitrile	0.23 (0.88)	0.41 (0.90)
Benzonitrile	0.47 (0.98)	0.056 (0.95)

[a] Potency of DNA damage was derived from calculated slopes by linear regression analysis for initial proportion of the dose–response curves from Tables 1 and 2.
[b] These values (r) mean the potency of linear regression.

3.2. Mutagenicity

The results of the mutagenicity conducted with and without S9 in Salmonella tester strains were presented in Table 4. 2-Aminofluorene (2-AF) and 4-nitroquinoline-N-oxide (4-NQO) was, respectively, used as the positive control for mutagen with and without metabolic activation. The maximum tested doses for each tested nitriles was chosen based on its solubility, and sub-toxic effect of this dose on bacterial growth. Results revealed that all five nitriles produce no mutagenicity (at least >two-fold background colonies) with and without metabolic activation.

4. Discussion

Genotoxins can elicit a variety of types of DNA damage, including base modification, DNA adduct, single-strand breaks, double-strand breaks, intra-or-inter-strand cross-link [21]. Single-cell gel electrophoresis (comet assay) was developed for the detection of these genotoxins. This assay has been widely used for different kinds of studies, including DNA repair [22–24], human biomonitoring [23,25], and genetic toxicology [24,26]. But there are few studies comparing the comet assay with other genotoxic tests. Furthermore, the detection of genotoxicity of nitriles using the comet assay was limited to few studies [27,28]. Thus, with the comet assay, we determined the genotoxicity of the tested nitriles before and after oxidation metabolism in lymphocytes and Hep G2 cells, respectively, after short time incubation (1 h). Hep G2 cells retained the characteristics of human origins and xenobiotic-metabolizing enzymes, enabling this type of cells to be a better model for reflecting the process in intact liver than other in vitro cells [29]. The rank order of genotoxic activity of nitriles in lymphocytes was acetonitrile > propionitrile > benzonitrile > methacrylonitrile > butyronitrile, while the rank order in Hep G2 cells was acetonitrile > butyronitrile > propionitrile > mehacrylonitrile > benzonitrile (Table 3). Unexpectedly, a high concentration of acetonitrile, propionitrile and benzonitrile caused the decreased DNA migration, indicating that this phenomena could be related with the formation of DNA-crosslinking adduct as shown in cisplatinum, mitomycin C and formaldehyde by the comet assay [30]. However, this still needs to be investigated. Furthermore, except for butyronitrile, the genotoxic activity of other four nitriles was more pronounced than that in Hep G2 cell. The reason for the difference of genotoxicity of these nitriles in lymphocytes and Hep G2 cells was unclear. It is well established that metabolism is a prerequisite for development of toxicity and carcinogenicity of aliphatic nitriles [2]. Although the information of the metabolism related with genotoxicity in these nitriles was unknown, the metabolism of acrylonitrile leading to genotoxicity was well documented. A highly significant correlation between gastric GSH levels and acrylonitrile-induced unscheduled DNA repair synthesis (UDRS) in DNA of gastric mucosal tissues was investigated [2], suggesting that homeostasis of gastric GSH may plays a major role in the initial processes underlying acrylonitrile-induced gastric carcinogenesis. On the other hand, binding of acrylonitrile and its reactive metabolites to tissue macromolecules such as nucleic acids may be responsible for its carcinogenicity in rats [2]. Acrylonitrile does not react directly with DNA very efficiently [31], whereas its epoxide intermediate, 2-cyanoethylene, has been shown to react with DNA *in vitro* [31,32]. The epoxide intermediate of methacrylonitrile, one of the tested nitriles in this study, was reported to be less reactive with DNA than that of acrylonitrile, which could be related with the hindrance of the methyl group in the epoxide intermediate of methacrylonitrile,

Table 4
The determination of mutagenicity induced by nitriles using Ames Salmonella strains.

Chemicals	His$^+$ revertants/plate[a]				
	Doses (μg/plate)	TA 98		TA 100	
		−S9	+S9.	−S9	+S9.
2-AF 4-NQO	1 10	ND[b] 2699 ± 1592.7	204.7 ± 1.5 ND	ND 2762.7 ± 295.8	424.3 ± 21.0 ND
Acetonitrile	3000	57.0 ± 2.8	47.0 ± 10.6	87.3 ± 2.1	139.7 ± 18.6
	1500	44.0 ± 7.0	32.7 ± 11.0	76.7 ± 11.0	135.3 ± 10.0
	1000	35.7 ± 7.0	47.0 ± 9.5	72.7 ± 18.9	117.0 ± 17.5
	500	28.7 ± 10.1	42.0 ± 14.1	73.7 ± 27.8	123.7 ± 10.7
	250	52.0 ± 6.1	43.7 ± 6.4	74.7 ± 13.5	147.0 ± 8.5
	0	32.0 ± 5.7	42.7 ± 18.5	87.0 ± 14.8	134.7 ± 11.6
Propionitrile	3000	29.0 ± 24.6	49.0 ± 14.7	85.3 ± 3.5	82.0 ± 18.5
	1500	27.7 ± 6.7	29.0 ± 8.7	76.0 ± 6.1	90.3 ± 2.1
	1000	31.0 ± 18.2	39.0 ± 10.0	71.3 ± 19.5	88.3 ± 6.5
	500	29.3 ± 4.0	37.0 ± 5.0	89.0 ± 9.8	83.3 ± 4.9
	250	28.7 ± 7.0	27.7 ± 4.5	99.0 ± 21.3	85.7 ± 9.0
	0	23.7 ± 1.2	25.3 ± 2.5	105.0 ± 16.1	88.3 ± 3.2
Methacrylonitrile	3000	35.8 ± 16.3	30.3 ± 6.1	95.3 ± 33.3	89.3 ± 7.6
	1500	32.3 ± 12.7	32.7 ± 5.8	108.7 ± 11.8	85.7 ± 15.0
	1000	29.0 ± 15.5	36.3 ± 19.7	90.3 ± 6.5	76.3 ± 3.1
	500	27.0 ± 13.5	31.0 ± 5.2	88.3 ± 9.0	71.0 ± 15.7
	250	22.7 ± 10.2	28.0 ± 1.7	96.3 ± 10.7	71.3 ± 4.0
	0	23.5 ± 10.9	30.0 ± 3.5	80.3 ± 20.6	73.3 ± 20.5
Butyronitrile	3000	16.0 ± 5.6	33.0 ± 10.0	105.0 ± 16.4	67.3 ± 4.2
	1500	14.3 ± 5.7	28.3 ± 11.9	103.3 ± 19.3	81.7 ± 6.0
	1000	13.7 ± 2.3	33.3 ± 13.6	87.7 ± 12.5	76.0 ± 3.5
	500	22.7 ± 2.1	26.0 ± 1.7	100.0 ± 7.0	87.0 ± 4.4
	250	17.0 ± 1.7	26.3 ± 5.5	97.7 ± 18.5	96.0 ± 2.0
	0	17.0 ± 4.6	29.6 ± 5.5	103.3 ± 17.7	82.3 ± 11.9
Benzonitrile	3000	18.0 ± 2.6	71.7 ± 6.7	25.3 ± 22.1	48.7 ± 11.0
	1500	14.7 ± 5.9	46.3 ± 6.8	69.3 ± 18.5	147.0 ± 22.7
	1000	27.3 ± 4.0	48.7 ± 9.0	79.7 ± 19.0	137.3 ± 2.9
	500	19.3 ± 4.5	64.0 ± 10.4	92.3 ± 11.2	140.3 ± 1.5
	250	15.7 ± 6.5	52.0 ± 9.8	84.7 ± 36.6	132.0 ± 11.5
	0	17.0 ± 2.6	44.6 ± 12.4	73.0 ± 28.2	138.0 ± 15.7

[a] Data represent the mean revertants ± S.D. of data from two independent experiments, each having three replicates ($n = 6$). The numbers of revertants that are > or = two-fold above that of negative control (without nitriles addition) are regarded as the positive response.
[b] ND, not done.

thus contributing to lower reactivity than that of acrylonitrile [2]. Like acrylonitrile, the metabolic products of acetonitrile and propionitrile could be access to DNA easily, thus causing the higher DNA-damaging potencies that that of methacrylonitrile as expected (Table 3). As in the case for methacrylonitrile, one benzyl group in benzonitrile could prevent this chemical from the oxidative metabolism in Hep G2 cells, which would result in the lowest reactivity of benzonitrile among all the tested nitriles. Noticeably, as shown in Table 3, only butyronitrile can cause stronger DNA-damaging effect in Hep G2 cells than in lymphocytes. The reasons for this result were unclear and seemed not be explained simply by the analysis of chemical analysis.

Taken together the results of our comet assay, we hypothesized that the metabolism of the tested nitriles in lymphocytes and Hep G2 cells appear to differ. However, the metabolic pathway leading to the genotoxicity of lymphocytes and Hep G2 cells still needs to be investigated, including the determination of the level of depletion of GSH, and DNA adduct in the lymphocytes and Hep G2 cells treated with these tested nitriles studied in this study.

A limited data regarding for the mutagenicity induced by these tested nitriles was consulted. Although the non-mutagenicity of acetonitrile or methacrylonitrile was proved by Jones et al. [6] or Nyska and Ghanayem [2], the mutagenicity of other three nitriles was unknown. In our study, no mutagenicity was found for all five nitriles with and without metabolic activation. The discrepancy of genotoxic results in the comet assay and the modified Ames test may be attributed to the different responses of eukaryotic cells and prokaryotic cells, separately, used in both tests.

Comparing the results obtained from both tests in this study, the comet assay seems to be more sensitive than the modified Ames test. Moreover, the comet assay used for the detection of all five nitriles as genotoxins in only one day, whereas the modified Ames test detected these nitriles as non-mutagens in two days. The comet assay would be a good tool for detecting the genotoxicity of these nitriles. Although the application of visual scoring method for comet assay would be questioned due to bias of each investigator, Collins et al. [33] indicated that visual scoring and undefined computer image analysis were equally useful human studies employing the comet assay. Also, a significant correlation between visual scoring results and percentage tail DNA was found in measuring the genotoxicity of North Sea marine sediment [34], and in analyzing DNA damage in canine and feline leukocytes [35]. In our previous paper, the comet assay with visual scoring was also applied to detect the genotoxicity of petroleum [18], benzidine and its derivatives [17], and *p*-phenylenediamine and its derivatives [36]. Nevertheless, it would be advisable for investigator unfamiliar with the comet assay to set up individual calibration curves correlating visual and computer image analysis score so that intra- and inter-investigator variation between comet measurements reduced to a minimum [35].

In conclusion, the present study was the first to compare these five nitriles for their ability to induce mutations in the modified Ames test after 30 min incubation and to induce genotoxicity in mammalian cells after 1 h incubation. The performing protocols for comet assay and modified Ames test including incubation time were according to the procedures of Chen et al. [18] and Bajpayee et

al. [20], respectively. Although the incubation time in both tests was different, the mutagenicity was not still observed using the modified Ames test after the prolonged incubation time up to 1 h (data not shown). All five nitriles were negative in the modified Ames test and positive in the comet assay. Further studies will be necessary to establish a link between the structures of these nitriles and their metabolic pathways leading to genotoxicity. Unlike the comet assay, some common cytogenetic methods such as chromosomal aberrations, micronuclei and sister-chromatid exchanges) detect the genotoxicity of proliferating cells only. These methods will be included in our experimental strategies to explore their feasibility in detecting the genotoxicity of these nitriles compounds in proliferating cells. These different end points detected from these cytogenetic methods and comet assay provide us with one clue for addressing the additional genotoxic mechanism of nitriles on the different growth phases of human cells.

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