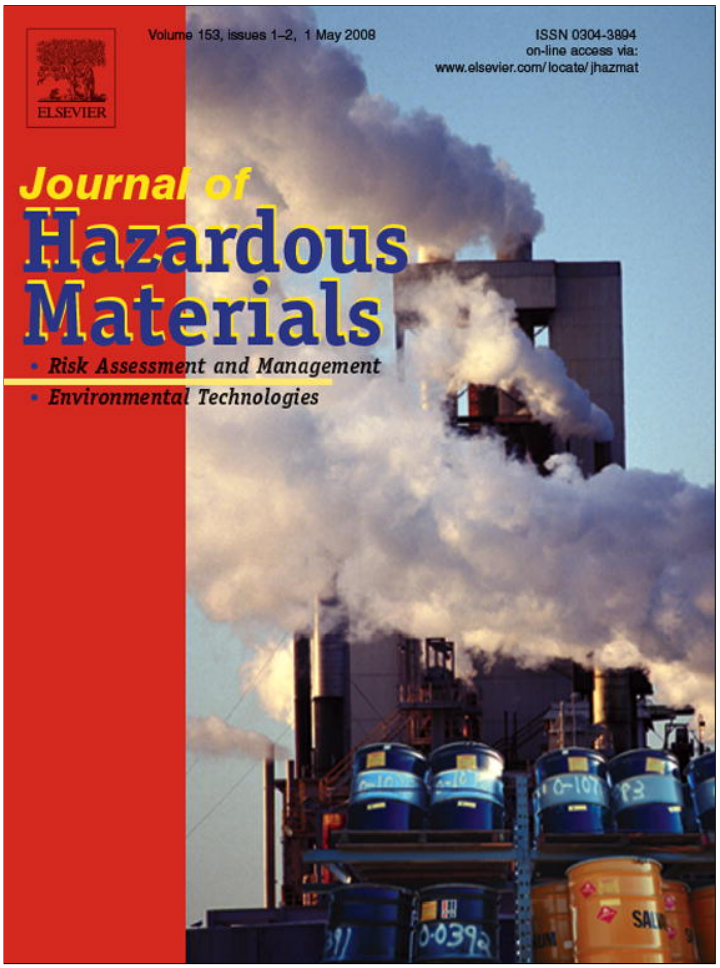

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Journal of Hazardous Materials 153 (2008) 784–791
Journal of Hazardous Materials
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Humic acid induced genotoxicity in human peripheral blood lymphocytes using comet and sister chromatid exchange assay

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Received 19 April 2007; received in revised form 5 September 2007; accepted 5 September 2007
Available online 8 September 2007

Abstract

Humic acid (HA) in well water used by the inhabitants for drinking is one of the possible etiological factors for blackfoot disease (BFD). Moreover, within BFD endemic areas cancers occur at significantly higher rates than in areas free of BFD. In this study, the genotoxic potential of HA is assessed using human peripheral blood lymphocytes. The cells were exposed to HA (0–200 μg/mL for 2 h), and the induction of DNA primary damage in cellular DNA was evaluated by single-cell gel electrophoresis (comet assay). HA-induced DNA damage was decreased by superoxide (O_2^-), hydrogen peroxide (H_2O_2), and reactive oxygen species (ROS) scavengers (superoxide dismutase, catalase, and Trolox), and nitric oxide (NO) synthase inhibitors (N^G-nitro-L-arginine methyl ester and N^G-methyl-L-arginine). Moreover, formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III), known to catalyze the excision of oxidized bases, increase the amount of DNA migration in HA-treated cells. Pretreatment of the cells with both the Ca^{2+}-chelator BAPTA and EGTA completely inhibited HA-induced DNA damage, indicating that HA-induced changes in Ca^{2+}-homeostasis are the predominant pathways for the HA induction of genotoxicity. Furthermore, sister chromatid exchange was found in the HA-treated lymphocytes. Our findings suggest that HA can induce oxidative DNA damage and genotoxicity in human lymphocytes.

Keywords: Humic acid; DNA damage; Sister chromatid exchange; Genotoxicity; Reactive oxygen species

1. Introduction

Blackfoot disease (BFD) is a peripheral arterial occlusive disease found in the inhabitants of the southwest coast of Taiwan in the 1970s [1,2]. Moreover, within the BFD endemic areas other diseases such as cancer (skin, lung, liver and bladder), cardiovascular anomalies, hypertension, diabetes mellitus, cerebral apoplexy, and goiter occur at significantly higher rates than in areas free of the disease [3,4]. Humic acid (HA) in well water used by the inhabitants for drinking is one of the possible etiological factors for BFD, however, the underlying pathophysiological mechanisms are still not established [5]. HA, a group of polymers with high-molecular weight derived from the decomposition of dead plants, is the most abundant in peat, soil and well water [6]. HA isolated from drinking-well water in BFD endemic areas is characterized by phenolic

Abbreviations: HA, humic acid; BFD, blackfoot foot disease; Comet assay, single-cell gel electrophoresis assay; ROS, reactive oxygen species; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radicals; SOD, superoxide dismutase; RNS, reactive nitrogen species; NO, nitric oxide; $OONO^-$, peroxynitrite; L-NAME, N^G-nitro-L-arginine methyl ester and; L-NMA, N^G-methyl-L-arginine; Fpg, formamidopyrimidine-DNA glycosylase; Endo III, endonuclease III; BHT, butylated hydroxytoluene; 1,10-PT, 1,10-phenanthroline; DTPA, diethylenetriaminepentaacetic acid; NC, neocuporine; EGTA, ethylene glycol-bis (amino-ethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; SCE, sister chromatid exchange; HFC, high frequency cells; As, arsenic.

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0304-3894/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jhazmat.2007.09.024

and phenolic–carboxylic polymers [7]. Epidemiological and geochemical studies have disclosed the presence of high concentrations of HA (approximately 200 ppm) in artesian well water from these areas [7], with daily intake by the average resident estimated to be as high as 400 mg [8,9]. Radioisotope tracing with iodinated HA in rats indicates that up to 60% of the ingested HA remains in the body 24 h after administration [10]. Unfortunately, to the best of our knowledge, no method is currently available to quantify the amount of HA in human blood and tissues.

We previously showed that interaction of HA with endothelial cells may directly increase their Ca^{2+} permeability, thus overstimulating the Ca^{2+} second messenger system [11]. Furthermore, HA has been shown to increase nitric oxide (NO)/peroxynitrite ($OONO^-$) production and apoptotic cell death in endothelial cells [12,13]. Our previous investigation indicated that HA induces echinocyte transformation via oxidative generation and a reduction in activities of the antioxidant enzyme in human erythrocytes [14,15]. Humic and fulvic acids extracted from podzol stimulate respiration in rat liver mitochondria resulting in the generation of intracellular reactive oxygen species (ROS) [16]. Therefore, we concluded that both oxidative stress and/or Ca^{2+} were mediators of cell toxicity caused by HA, and that delineation of the precise pathways for HA cytotoxicity needs further characterization [11,12].

ROS can damage different cellular macromolecules, including DNA, which is directly responsible for mutation and carcinogenesis. However, HA has mainly been studied for possible antimutagenic activity because of its metal chelating properties, such as mutagen arsenic (As) [17–20]. HA genotoxicity has not been well demonstrated either *in vitro* or *in vivo*. In this study, therefore, the genotoxic potential of HA in human peripheral blood lymphocytes was assessed using single-cell gel electrophoresis assay (comet assay). DNA migration increase was used as indicators of HA toxicity because they are closely related to mutagenicity and clastogenicity. In this *ex vivo* system, we also tested the DNA-damaging protective activity of different, well-known compounds over a wide range of concentrations. Furthermore, sister chromatid exchange (SCE) was examined in HA-treated lymphocytes. These results may provide a possible mechanism leading to carcinogenesis and atherosclerosis in BFD endemic regions.

2. Materials and methods

2.1. Preparation of synthetic HA

To better define the chemical components associated with the adverse effects assumed to result from the consumption of contaminated artesian well water, synthetic HA was synthesized from monomeric protocatechuic acid and, thus free of other inorganic contaminants, was used for this study according to the published procedure, with slight modifications as described previously [21]. For oxidative polymerization, 1 g of protocatechuic acid in 100 mL of distilled water was oxidized with sodium periodate for 24 h in a water bath at 50 °C with shaking. After centrifugation at 3000 × *g*, the supernatant was acidified to pH 1.0 with 0.1 N HCl. The acidified solution was again centrifuged, and the precipitate was treated with 0.1 N NaOH to solubilize the HA. The HA was further purified using absorption chromatography with XAD-7 resin and fractionated by Sephadex G-25 chromatography, as described previously [11]. The HA solution was ultrafiltered through a Molecular/Por membrane (which excludes particles of <500 Da MW). The resultant HA (with MWs of 500 Da to several tens of thousands of Daltons) was collected for use in this study.

2.2. Human mononuclear cell preparation

Cells were isolated from the freshly donated peripheral blood of healthy volunteers as previously described by Aliverti et al. [22]. The local institutional review committee approved the research proposal and informed consent was obtained from all individuals involved in the study. Briefly, blood was immediately mixed with 5% EDTA in 0.9% NaCl to a ratio of 9:1 to avoid coagulation, and then diluted 1:1 with phosphate-buffered saline (PBS). The mononuclear cell population was subsequently separated by density centrifugation over a layer of Histopaque (450 × *g* for 10 min at 18 °C). The cells in the band over the Histopaque were carefully collected, washed two more times in cold PBS and centrifuged (450 × *g* for 10 min at 4 °C) and resuspended in RPMI 1640 cell culture medium. The calcium concentration of the medium was 0.4 mM. Finally, the cells were counted under a microscope with a hemocytometer and adjusted to 1.5×10^6 cells/mL prior to use.

2.3. Single-cell gel electrophoresis assay (comet assay)

The assay was essentially the same as that described by Singh et al. [23] with some modifications. Isolated lymphocytes were incubated with the indicated amount of HA (0–200 μg/mL) for various times (0–2 h) at 37 °C depending on the experiment. Cells were suspended in 1% low-melting-point agarose in PBS (pH 7.4) and pipetted onto superfrosted glass microscope slides precoated with a layer of 1% normal-melting-point agarose (warmed at 37 °C prior to use). The agarose was allowed to set at 4 °C for 10 min, and then the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10], 1% Triton X-100) at 4 °C for 1 h. Slides were then placed in single rows in a 30-cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA (pH 13.4) at 4 °C for 40 min to allow separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm), 300 mA for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris (pH 7.5) at 4 °C before staining with DAPI (5 mg/mL). DAPI-stained nucleoids were examined under a UV microscope using a 435 nm excitation filter at 200× magnification.

2.4. Image analysis and scoring

The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected nucleoids. DNA damage in the SMCs,

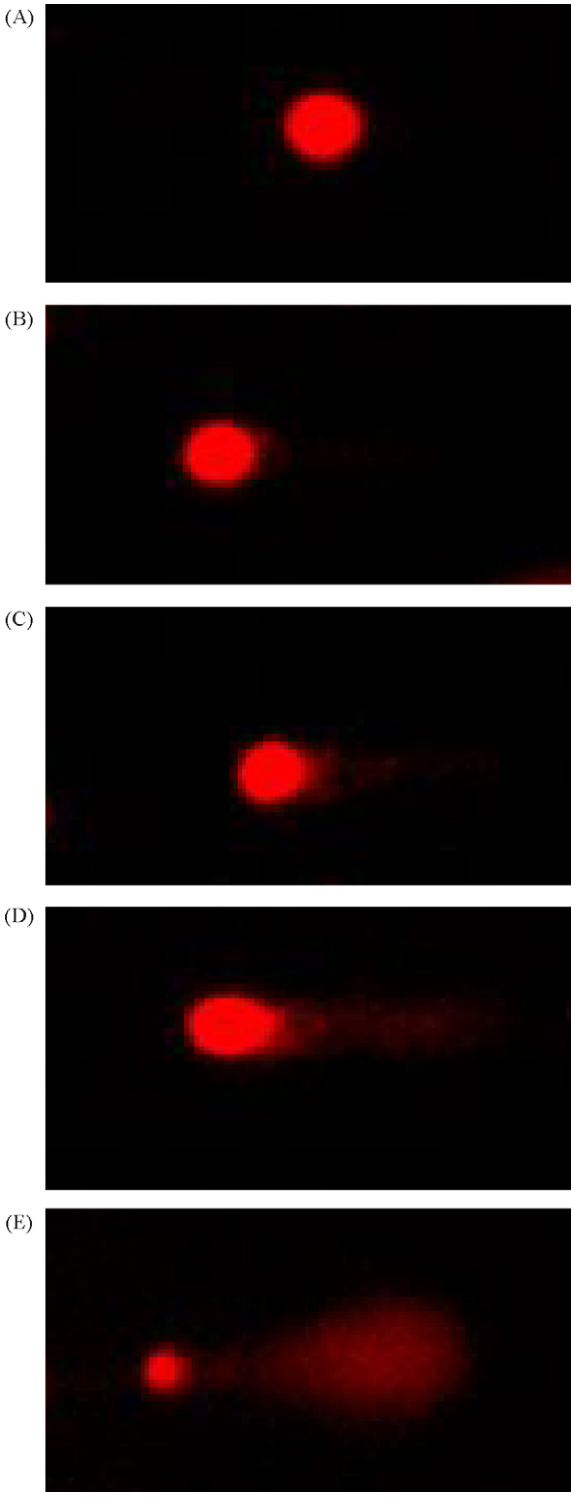

Fig. 1. DNA damage, detected by comet assay and measured as reported in See Section 2, in human lymphocytes treated with HA (200 μg/mL for 2 h). The cellular DNA was stained with DAPI and photographed in a fluorescence photomicroscope. (A) class 0; (B) class 1; (C) class 2; (D) class 3; (E) class 4.

as DNA strand breaks including double and single-strand variants at alkali-labile sites, was analyzed in an alkaline condition (pH 13.4). The comet-like DNA formations were categorized into five classes (0, 1, 2, 3 or 4) representing increasing DNA damage seen as a "tail" (Fig. 1). Each comet was assigned a value according to its class. Accordingly, the overall score for 100 comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). In this way, the overall DNA damage of the cell population can be expressed in arbitrary units [24]. Observation and analysis of the results were always performed by the same experienced person. The analysis was blinded, with the observer having no knowledge of slide identity.

2.5. Effect of Fpg and Endo III on HA-induced DNA damage

Isolated lymphocytes were treated with 25 or 50 μg/mL HA for 2 h. Cells were suspended in 1% low-melting-point agarose in PBS and pipetted onto superfrosted glass microscope slides. For enzyme digestion, slides were washed with distilled water after lysis and then incubated with enzyme reaction buffer (100 mM NaCl, 20 mM Tris, 10% glycerol, 0.5 mM EDTA, and 1 mM DTT, pH 7.6) [25,26]. Fpg and Endo III (1 U/mL) were then added to the incubation mixture. A coverslip was applied, and the mounted slides were placed in a sealed box containing a piece of wet tissue paper to maintain moisture, and the box was incubated at 37 °C for 2 h. At the end of enzyme digestion, these slides were washed, and alkaline denaturation and electrophoresis were performed following the procedures described above.

2.6. Effect of ROS/RNS modulators and iron/calcium chelating agents on HA-induced DNA damage

Isolated lymphocytes were pretreated with ROS/RNS modulators (SOD, Catalase, Trolox, L-NAME, L-NMA, Vit E, and BHT) or iron/calcium chelating agents (1,10-PT, DTPA, NC, EGTA, and BAPTA) at the indicated doses about 30 min prior to the addition of HA. Subsequently, the cells were incubated with 200 μg/mL HA for 2 h. Comet assay was then conducted following the procedures described above.

2.7. Sister chromatid exchange

Human lymphocytes were analyzed using sister chromatid exchange (SCE) assay, a sensitive indicator of chromosome damage [27]. Cells were isolated from the freshly donated peripheral blood of healthy volunteers and whole blood was drawn into heparinized tubes. Lymphocyte cultures were set up by adding 0.2 mL of heparinized whole blood to 4.8 mL of RPMI 1640 medium with 15% fetal bovine serum and 1% penicillin/streptomycin. Lymphocytes were stimulated by 3% phytohemagglutinin. For the SCE demonstration, 40 μM bromodeoxyuridine was added at the beginning of the culture period. The cells were incubated in the dark for 68 h at 37 °C and then 2 h before fixation, 2×10^{-7} M colcemid was added to arrest the chromosomes at metaphase. The cells were treated with the indicated amount of HA (0–200 μg/mL) during the last 24 h. Cells were then collected by centrifugation, swollen in 0.075 M KC1 for 10 min at 37 °C, and fixed

in ice-cold methanol:acetic acid (3:l). Drops of cell suspension were placed on slides. The cells were stained for SCE analysis using the fluorescence plus Giemsa method. SCEs of lymphocytes were microscopically examined and counted from photographs. All slides were scored by the same person. For the observation of SCEs per cell, at least 25 s division metaphases were scored per sample. High frequency cells (HFC) were determined as the percentage of lymphocytes exhibiting an SCE score over the median 95% value of the result from the HA-untreated cells [27]. Simple linear regression was used to calculate the dose–response regression curve of HA-treated lymphocytes.

2.8. Statistical analysis

Data are presented as mean ± S.E. Analysis of variance (ANOVA) followed by Dunnett's test for pairwise comparison was applied for evaluation of statistical significance, which was defined as $P < 0.05$ for all tests.

3. Results

3.1. Effects of HA treatment on DNA damage

Isolated human lymphocytes were exposed to HA and the induction of DNA damage in the cellular DNA was evaluated using single-cell gel electrophoresis (comet assay) (Fig. 1). Lymphocytes were treated with various concentrations of HA (25–200 μg/mL) for 0.5, 1 or 2 h. Results indicated that enhanced DNA damage was clearly detected in lymphocytes after HA treatment in a time and dose-dependent manner (Fig. 2).

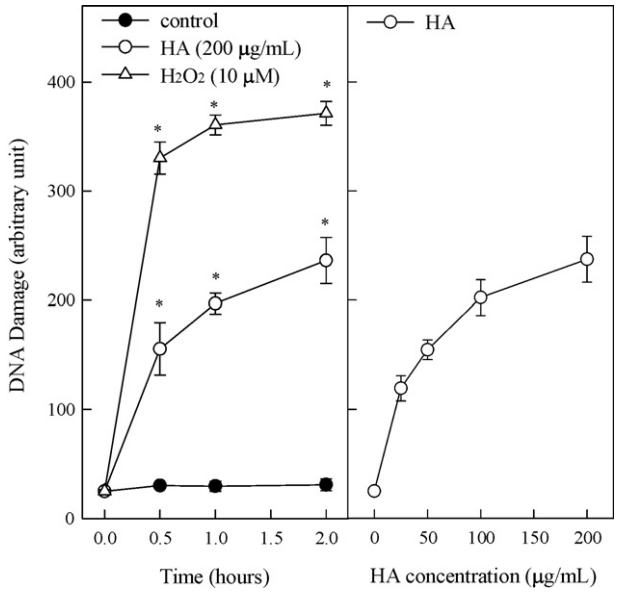

Fig. 2. DNA damage in human lymphocytes treated with HA for different times and different concentrations (2-h treatment). H_2O_2 (10 μM) was used as a positive control. Results are the mean ± S.E. of three assays. (*) Statistically significant difference compared to untreated cells ($P < 0.05$).

3.2. Effects of ROS and RNS modulators on HA-induced DNA damage

As genotoxic involvement was demonstrated for ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^\bullet$OH), and reactive nitrogen species (RNS), including NO and $OONO^-$, the effects of their modulators on HA-induced DNA damage (genotoxicity) with 2-h HA treatment (200 μg/mL) were studied. Results show that superoxide dismutase, catalase and Trolox (scavengers of O_2^-, H_2O_2 and ROS, respectively), N^G-nitro-L-arginine methyl ester/N^G-methyl-L-arginine (L-NAME and L-NMA, NO synthase inhibitors) effectively reduced HA-induced DNA damage (Fig. 3A and B). Vitamin E and BHT for lipid peroxidation did not offered significant protection in this system (Fig. 3C).

3.3. Effects of Fpg and Endo III protein on HA-induced DNA damage

The above results suggest that ROS/RNS are involved in HA-induced DNA damage. We then asked if oxidative DNA damage is involved. *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III) were then used to determine whether oxidized oligonucleotide was induced in HA-treated lymphocytes [25,26]. Fpg and Endo III are known to catalyze the excision of oxidized bases such as formamidopyrimidine and 8-oxoguanine; therefore, they will convert these oxidized bases into DNA strand breaks. In this study, it was clearly shown (Fig. 4) that Fpg and EndoIII incubation of the lymphocytes treated with HA (25 and 50 μg/mL for 2 h) increases the sensitivity of the comet assay to detect DNA damage. We may conclude that the HA treatment induced a strong oxidative DNA damage.

3.4. Involvement of intracellular iron in HA-induced DNA damage

It has been proposed previously that intracellular iron, which is able to catalyze the formation of $^\bullet$OH by Fenton-type reactions, plays a crucial role in H_2O_2-induced DNA damage [28]. Therefore, the role of iron in HA (200 μg/mL)-induced DNA damage in 2-h treatment of lymphocytes was investigated (Fig. 5A). Pre-incubation with the lipophilic (specific) ferric ion chelator, 1,10-phenanthroline (1,10-PT), inhibited DNA damage in a dose-dependent manner (2 h) (Fig. 5A). Diethylenetriaminepentaacetic acid (DTPA), a membrane impermeable iron chelator, was ineffective (Fig. 5A). None of the iron chelators above produced DNA damage by themselves. In contrast, the specific cuprous ion chelator, neocuproine (NC, 2,9-dimethyl-1,10-phenanthroline), did not offer protection in this system (Fig. 5A). In fact, membrane-permeable copper chelating agent induced DNA damage by itself [28]. These results suggest that intracellular redox-active iron, but not copper, is involved in HA-induced DNA damage in lymphocytes.

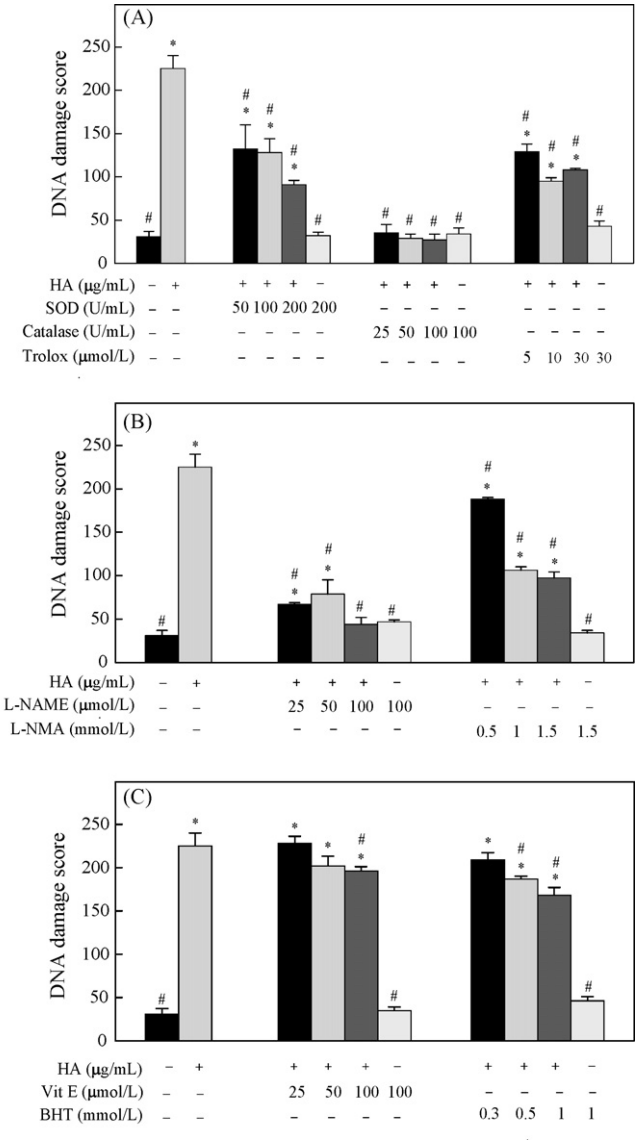

Fig. 3. Effects of ROS/RNS modulators on HA-induced DNA damage in human lymphocytes (200 μg/mL HA, 2 h). The cell pre-treatment (see Section 2) was performed with different doses of the following: (A) SOD, catalase, and Trolox; (B) L-NAME and L-NMA; and (C) vitamin E and BHT. DNA damage was assessed by alkaline comet assay and measured as reported in Section 2. Modulators were added to the incubation mixture 30 min prior to the addition of HA. Results are the mean ± S.E. of three assays. (*) Statistically significant difference compared to untreated cells ($P < 0.05$). (#) Statistically significant difference compared to cells treated with HA alone (200 μg/mL) ($P < 0.05$).

3.5. Role of calcium in HA-induced DNA damage

Experiments were conducted to assess the effects of extracellular calcium. Such experiments are typically conducted in calcium-free medium with the addition of a calcium chelator such as EGTA (ethylene glycol-bis (amino-ethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid, 0.1–0.4 mM). Results indicate that pretreatment of the cells with the Ca^{2+}-chelator EGTA effectively inhibited HA (200 μg/mL)-induced DNA damage with 2-h treatment of lymphocytes (Fig. 5B). In this study, the Ca^{2+}-chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) was also used to bind intracellular

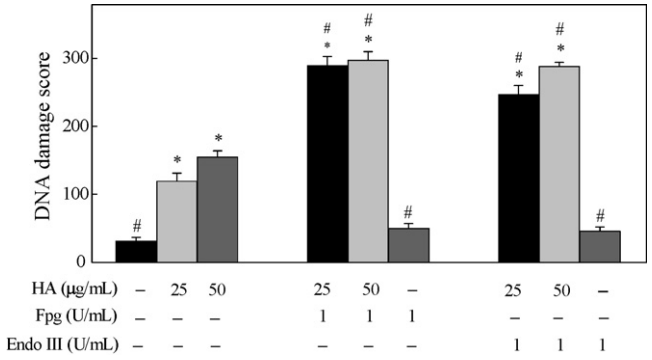

Fig. 4. DNA oxidative damage, detected by the modified comet assay protocol (with/without Fpg and Endo III), in lymphocytes treated with 25 or 50 μg/mL HA for 2 h (see Section 2). Results are the mean ± S.E. of three assays. (*) Statistically significant difference compared to untreated cells ($P < 0.05$). (#) Statistically significant difference compared to cells treated with HA alone (25 or 50 μg/mL) ($P < 0.05$).

calcium, which is at much lower concentrations than extracellular calcium. We observed that BAPTA, at low concentrations (50–200 μM), completely inhibited HA (200 μg/mL)-induced DNA damage with 2-h treatment of lymphocytes (Fig. 5B). The present findings suggest that the involvement of both intra and extra-cellular Ca^{2+} changes (calcium influx and intracellular calcium overload) in HA-induced DNA damage of lymphocytes.

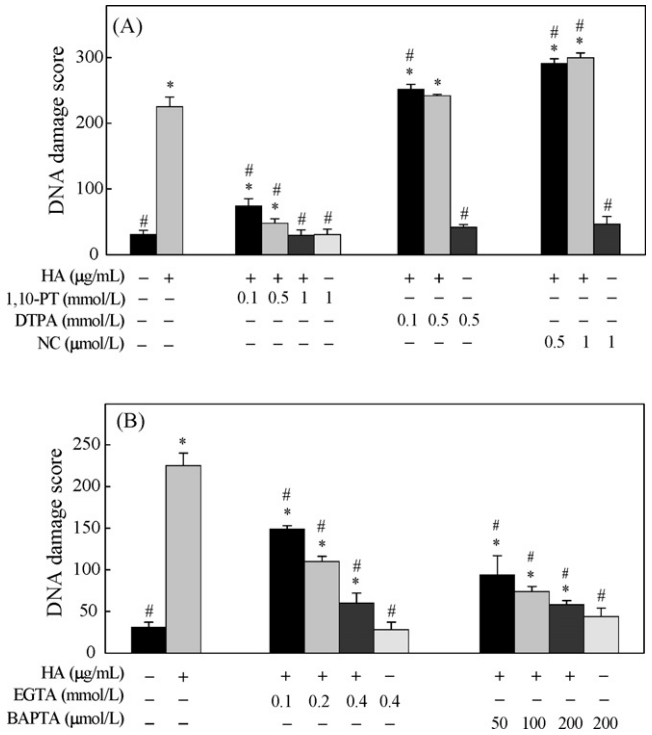

Fig. 5. Effects of iron/calcium chelating agents on DNA damage induced in lymphocytes treated with 200 μg/mL HA for 2 h. The cell pre-treatment (see Section 2) was performed with different doses of the following: (A) 1,10-PT, DTPA, and NC; (B) EGTA and BAPTA. Modulators were added to the incubation mixture 30 min prior to the addition of HA. Results are the mean ± S.E. of three assays. (*) Statistically significant difference compared to untreated cells ($P < 0.05$). (#) Statistically significant difference compared to cells treated with HA alone (200 μg/mL) ($P < 0.05$).

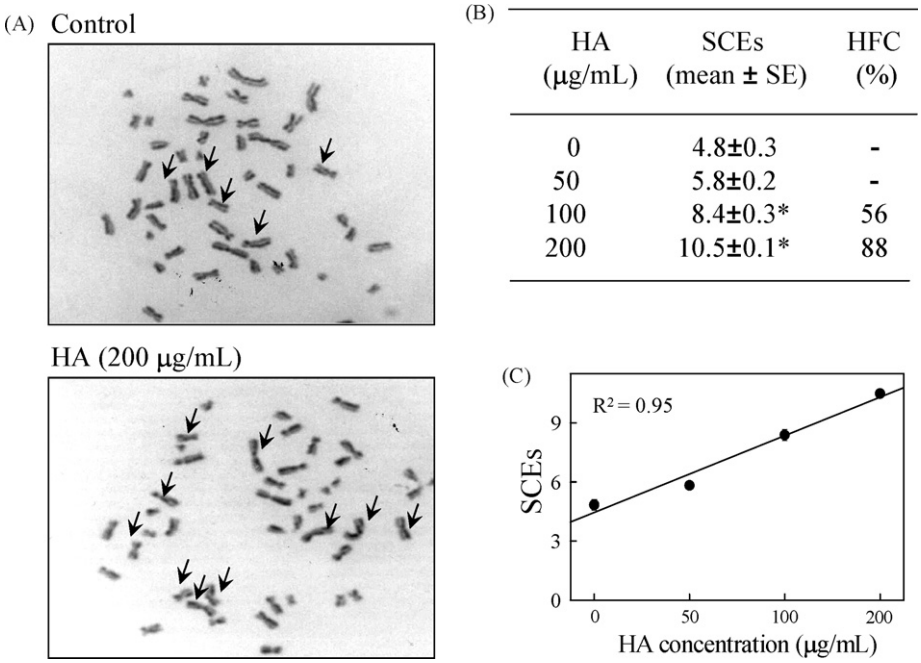

Fig. 6. SCE induction by HA in lymphocytes. Lymphocytes were incubated with 0, 50, 100 or 200 μg/mL HA for 24 h. (A) The photographs are images of untreated cells and cells treated with 200 μg/mL HA for 24 h. (B) Data are presented as the number of SCEs per cell and the percentage of high frequency cells (HFC%). Results are the mean ± S.E. of three assays. (*) Statistically significant difference compared to untreated cells ($P < 0.05$). (C) The dose–response curve for HA induction of SCEs in lymphocytes. The regression line was calculated by simple linear regression.

3.6. Induction of SCEs by HA

In this study, endpoints of genotoxicity, such as SCEs, were examined following *in vitro* HA treatment. We found that treatment with HA (50–200 μg/mL) for 24 h produced significant increases in the SCE frequencies and high frequency cells (HFC) in a dose-dependent manner over the analogous levels in untreated lymphocytes (Fig. 6).

4. Discussion

The results of this study suggest that HA genotoxicity in human lymphocytes is a consequence of induction of DNA damage and sister chromatid exchange. HA-induced DNA damage in lymphocytes was prevented by treatment with various modulators, suggesting that ROS and/or RNS production, and elevation of the intracellular calcium concentration were required as common signals for HA induction of genotoxicity. The results of the modulator experiments are summarized in Table 1. The O_2^- is converted by superoxide dismutase to H_2O_2, which gives rise to $^{\bullet}OH$, which are potent DNA-damaging agents in the presence of Fe^{2+} [28]. Formation of $^{\bullet}OH$ close to DNA results in damage to the molecular chain, including base modifications, DNA strand breaks, and sister chromatid exchange. HA-increased NO may also react with O_2^- to produce peroxynitrite and cause DNA damage [29]. Peroxynitrite, an oxidizing and nitrating agent, provides another route for the formation of hydroxyl radicals, and it is capable of inducing DNA oxidation [30]. In conclusion, HA induces oxidative DNA damage via two pathways: O_2^-, H_2O_2 and $^{\bullet}OH$; and, NO, O_2^- and $OONO^-$.

DNA damage has also been linked to intracellular Ca^{2+} increases, indicating an obligatory intermediary role for Ca^{2+} [31,24]. Previously, we had demonstrated that HA disrupts intracellular calcium homeostasis and HA enhances the permeability of cell membranes to extracellular Ca^{2+}, resulting in sustained elevation of cytosolic Ca^{2+} in endothelial cells. The basal [Ca^{2+}] level of the resting cells measured just after Fura-2 (calcium indicator) loading was 70 ± 5 nM and for endothelial cells treated

Table 1
Modulation of HA-induced DNA damage in human lymphocytes

Agent	Putative function	HA[b]
SOD (200 U/mL)[a]	↓ O_2^- (outside cells)	↓↓
Catalase (100 U/mL)	↓ H_2O_2	↓↓↓
Trolox (30 μmol/L)	↓ ROS	↓↓
L-NAME (100 μmol/L)	↓ NO	↓↓↓
L-NMA (1.5 mmol/L)	↓ NO	↓↓
Vit. E (100 μmol/L)	↓ lipid peroxidation	=
BHT (1.0 mmol/L)	↓ lipid peroxidation	=
1,10-PT (0.5 mmol/L)	↓ Fe	↓↓↓
DTPA (0.5 mmol/L)	↓ Cu	=
NC (1.0 μmol/L)	↓ Cu	=
EGTA (0.4 mmol/L)	↓ [Ca^{2+}] out	↓↓↓
BAPTA (200 μmol/L)	↓ [Ca^{2+}] in	↓↓↓

Data are from Figs. 3 and 5.

[a] Represents the most effective concentration.

[b] Effectiveness of a modulator. '=', '↑or↓', '↑↑ or ↓↓' and '↑↑↑ or ↓↓↓' represent an increase or decrease of <25%, 25–50%, 50–75% and >75%, respectively. The effectiveness was calculated by the formula: [HA plus modulator DNA damage score/(HA DNA damage score + modulator DNA damage score − untreated cell DNA damage score)] × 100 with overall DNA damage of the cell population expressed in arbitrary units.

with HA (100 and 200 μg/mL) the maximum [Ca^{2+}] level rose to, respectively, 130 nM and 180 nM 25 min subsequent to exposure [11]. It appears reasonable to propose, therefore, that the initial events during HA treatment lead to a perturbation of cell calcium homeostasis prior to occurrence of DNA damage in lymphocytes since the exclusion of intra and extra-cellular Ca^{2+} by chelator BAPTA and EGTA strongly inhibited HA-induced DNA damage. Our findings suggest that HA-induced changes in Ca^{2+}-homeostasis (calcium influx and intracellular calcium overload) are the predominant pathways for the HA induction of genotixicity.

It has been proposed that dissolved HA, being amphiphilic in nature, forms regions similar to detergent micelles in aqueous solution [32]. Dissolved detergent-like HA can enhance the solubility of hydrophobic species involving the hydrophobic microenvironment of the pseudomicellar interior of HA [33,34]. We suggest, therefore, that lymphocyte interaction with the synthetic HA, probably forming pseudomicelles, may directly increase their Ca^{2+} permeability, thus overstimulating the Ca^{2+} second messenger system through activation of Ca^{2+}-dependent enzymes and inducing subsequent genotoxicity. Although HA is known to interact with hydrophobic environments, the nature and extent of these interactions are not fully understood. Further investigation is required, therefore, to identify the mechanism/s responsible for the structural changes in HA and/or the HA-membrane interface and therefore, Ca^{2+} permeability.

These genotoxicity endpoints require a complex concatenation of cellular processes and are resolved at time points several hours after the HA initiation. An *in vivo* study showed that HA induces chromosomal abnormalities in intestinal cells and marginal, non-significant induction of aneuploidy in bone marrow cells [35]. The cytogenetic effects of HA exposure provide evidence that it acts as a clastogenic/aneugenic carcinogen in mammalian cells. Interestingly, increased frequencies of SCEs and chromosomal aberrations have been observed in lymphocytes isolated from cancer patients living in areas endemic for BFD [36]. However, HA (200 μg/mL) did not cause mutation in *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 evaluated by the Ames test (data not shown). According to these results we can assume that the comet assay was the most sensitive assay, followed by the SCE test. The Ames test does not appear to be sensitive enough for HA genotoxicity. Similarly, it has been recently reported that the comet assay, but not the Ames test, can detect the genotoxicity of water soil leaches [37]. Further study is needed to determine whether the difference in metabolism of HA in eukaryotic cells and prokaryotic cells leads to opposite results in genotoxicity tests, as evaluated by comet assay and Ames test, respectively.

HA and arsenic (As) in the well water consumed by the inhabitants are the two major potential etiological factors. The debate with respect to the cause of BFD in Taiwan remains unresolved [38]. HA, which is stable to heat and acid–base action, contains carboxyl and hydroxyl as the main functional groups and exists as an organometallic complex containing inorganic elements such as As and other metals. Investigation indicates that HA-As^{5+} (33–44%), HA-As^{3+} (3–13%) and As_2O_5 (53–54%) are the main As species in ground water from BFD endemic areas [39]. Because of its metal chelating properties, the HA content in artesian well water in BFD endemic areas has a positive linear correlation with As concentration ($r = 0.49$, $P < 0.001$) [40]. It appears likely, therefore, that both As and HA in the well water have a direct relationship with epidemical BFD. Further, liver cancer mortality has been correlated positively with HA levels in BFD endemic areas [41].

The epidemiological evidence for shared risk factors for cancer and atherosclerosis has been reviewed [42]. Exposure to carcinogenic environmental agents is associated with an increased risk of atherosclerosis. As increasing DNA damage is correlated with the severity of atherosclerotic disease [43], it is tempting to speculate that HA may induce human vascular disorders associated with BFD via oxidative DNA damage. However, although ROS and RNS are known to damage DNA, proteins and lipids, the HA-induced oxidative stress may also cause human disorders via pathways other than oxidative DNA damage. Much effort has been devoted to elucidating the molecular basis for the carcinogenic potentials of HA; however, at the time of writing, no mechanism has been unequivocally established.

5. Conclusions

This work suggests that: (i) HA can induce genotoxicity in human lymphocytes as evidenced by DNA damage and SCE data; (ii) HA-induced changes in Ca^{2+}-homeostasis and ROS/RNS production appear to be the main pathways of genotoxicity induction. Since, it is generally accepted that the loss of genomic stability is one of the most important aspects of carcinogenesis, this demonstration of HA genotoxicity leads to hypothesis that HA could induce cancer by disrupting genetic integrity, although the mechanism for genotoxicity seems to be less complicated than its putative carcinogenic analog. Our findings suggest that HA genotoxicity ability may be one of the underlying mechanism for carcinogenesis and atherosclerosis observed in the BFD endemic region. Thus, HA may be etiologically significant for human cancers and vascular disorders in Taiwan's BFD endemic areas. However, further investigations on HA activity, both *in vitro* or *in vivo*, are necessary to elaborate and exploit this heuristic potential.

Acknowledgments

This work was supported by NSC94-2320-B-166-005 and NSC93-2320-B-166-0039 from the National Science Council of the Republic of China.

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