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Suppressive Effects of Various Antioxidants on Melamine-induced Oxidative DNA Damage in Human Lymphocytes

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Accepted 13 August 2009

Abstract

Melamine, which is used to produce melamine resin for various industrial applications, has a high nitrogen content by mass. For this reason, it has been illegally added to foods to increase their apparent protein content. In the present investigation, melamine-induced oxidative damage of human lymphocyte DNA was evaluated by Comet assay. The *in vitro* oxidative DNA damage caused by melamine increased in a dose-dependent manner. This DNA damage was significantly inhibited by treatment with ascorbate. Moreover, the traditional Korean medicinal herb, named *Acanthopanax*, red ginseng and green tea markedly reduced the DNA damage. Various edible plant extracts also inhibited melamine-induced oxidative DNA damage *in vitro*. Melamine enhanced intracellular ROS generation, and this effect was suppressed by treatment with various antioxidants.

Keywords: Melamine, Comet assay, Oxidative DNA damage, Reactive oxygen species (ROS), Antioxidant

Melamine (1,3,5-triazine-2,4,6-triamine) consists of an organic base and a cyanamide trimer, with a 1,3,5-triazine skeleton. The combination of melamine and formaldehyde produces melamine resin, which has been used in Formica and cleaning products[1]. Owing to its high nitrogen content, melamine was once considered for use as a crop fertilizer, but its slow mineralization and high cost precluded its use as fertilizer[2]. Furthermore, melamine is not a useful nitrogen source for ruminants, because of its slow and incomplete hydrolysis in cattle[3]. Although not approved as a food additive for use in human or animal feed, melamine has been illegally added to some food products, because its high nitrogen content skews the protein analysis, making the food appear to have a higher protein content[4]. However, when absorbed into the bloodstream, melamine becomes concentrated and interacts with cyanuric acid in the renal microtubules, forming large numbers of crystals that damage renal cells, resulting in kidney malfunction. A pet food recall was initiated in 2007 because melamine contamination caused renal failure in hundreds of cats and dogs[5]. Chronic ingestion of melamine can also result in reproductive damage, bladder or kidney stones, and possibly bladder cancer[6]. Melamine has a reported oral LD_{50} of 3,161 mg/kg in rats and a reported dermal LD_{50} of 1,000 mg/kg in rabbits[7].

The single-cell gel electrophoresis assay (comet assay) is a well-established genotoxicity test for estimating DNA damage at the individual cell level, both *in vivo* and *in vitro*. It has been widely used to detect primary DNA damage in human and animal cells exposed to various noxious substances relevant to environmental or occupational exposure[8,9]. The comet assay has been applied to species used in biomonitoring or toxicity testing and has proven to be a sensitive system for genotoxicity screening[10-12]. Comet assays are also useful for detecting DNA damage induced by oxidative damage caused by the generation of free radicals[13].

In this study, melamine-induced DNA damage in human lymphocytes was examined by comet assay and ROS quantification. The ability of various antioxidants to suppress this oxidative damage of human lymphocyte DNA was also investigated.

Melamine-induced Oxidative DNA Damage

Human lymphocytes treated with 200 μM to 50 mM melamine exhibited DNA damage, as determined by the olive tail moment in a comet assay (Figure 1). Melamine at low concentrations did not cause significant DNA damage, whereas high concentrations of melamine induced severe DNA damage. The olive tail moment at 50 mM melamine was about 33.41 ± 0.46, compared with 9.03 ± 1.23 in the PBS-treated control,

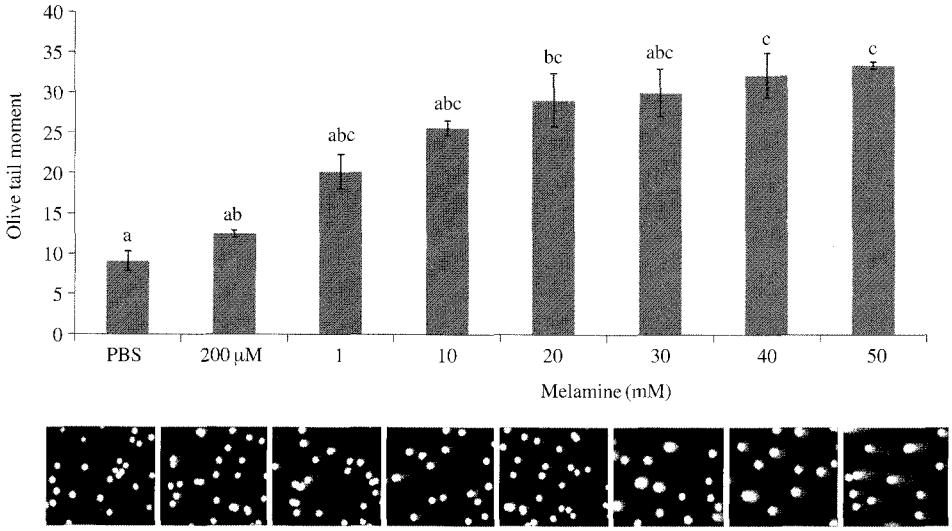

Figure 1. Melamine-induced oxidative DNA damage in human lymphocytes.

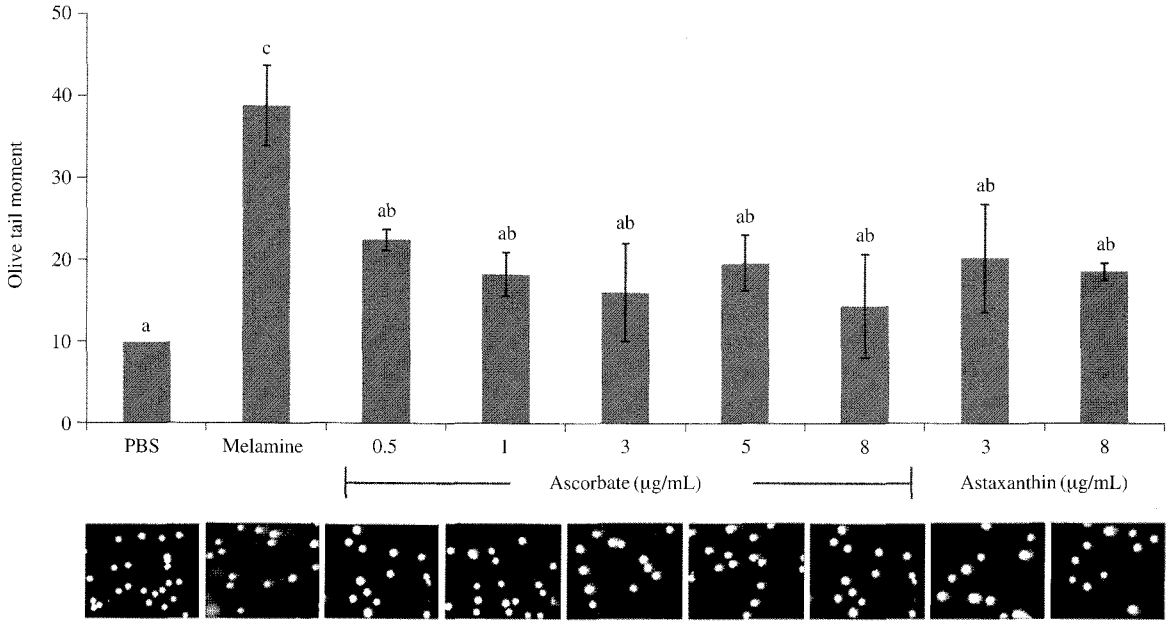

Figure 2. Suppressive effects of ascorbate and astaxanthin on the melamine-induced oxidative DNA damage in human lymphocytes.

indicating an approximately 4-fold increase in DNA damage at 50 mM melamine. Hydrogen peroxide is thought to cause DNA strand breakage by generating hydroxyl radicals close to the DNA molecule[14]. At 150 μM, H_2O_2 showed olive tail moment of about 30, which was an approximately 6-fold increase in DNA damage in comparison with the control[15]. These results indicate that melamine-induced oxidative DNA damage is less severe than that induced by H_2O_2.

Suppressive Effects of Antioxidants on Melamine-induced Oxidative DNA Damage

The addition of ascorbate inhibited the oxidative DNA damage caused by 50 mM melamine, as demonstrated by a reduction of the olive tail moment in a comet assay (Figure 2). Increases in the ascorbate concentration did not enhance the suppression. Astaxanthin, a strong antioxidant, also protected the lymphocytes from DNA damage induced by 50 mM melamine (Figure 2).

Figure 3 shows the effects of the traditional Korean medicinal agents *Acanthopanax* and red ginseng on melamine-induced DNA damage *in vitro*. Polyphenol extracted from Korean green tea was used as a positive control, and there was no significant difference in the olive tail moment between polyphenol-treated and PBS-treated lymphocytes. Korean *Acanthopanax* and red ginseng extract as well as polyphenol markedly reduced the oxidative DNA damage in human lymphocytes.

Next, the effects of edible plant extracts on melamine-induced oxidative DNA damage were examined. Lymphocytes treated with the extracts of paprika, broccoli, grape, lettuce, and garlic showed marked reductions in melamine-induced oxidative DNA damage (Figure 4). These plants have been shown to contain a variety of phytochemicals, including polyphenols, carotenoids, and flavonoids, which are known to be powerful natural antioxidants. Thus, the antioxidant activities of these plants may account for their inhibition of oxidative DNA damage.

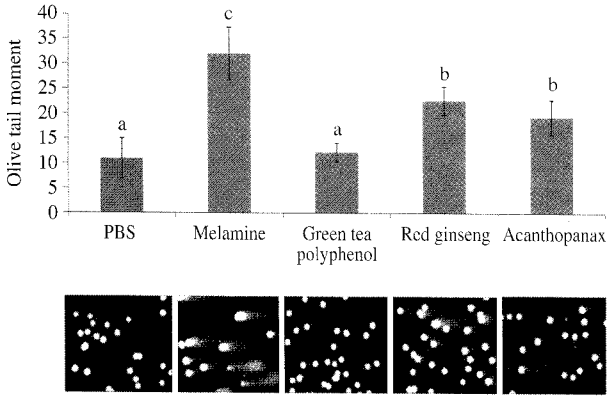

Figure 3. Suppressive effects of traditional Korean medicinal herbs on the melamine-induced oxidative DNA damage in human lymphocytes.

Electrolyzed-reduced water has recently been shown to be a powerful radical scavenger[16]. As shown in Figure 5, PBS prepared from electrolyzed-reduced water alone reduced the melamine-induced lymphocyte DNA damage to a level that was not significantly different from that in the PBS-treated controls.

Sodium benzoate is a food preservative and is used most often in acidic foods. The possibility that sodium benzoate may synergistically enhance melamine-induced DNA damage was examined. Sodium benzoate alone caused oxidative DNA damage in lymphocytes, in a dose-dependent manner (Figure 6A). The addition of sodium benzoate synergistically enhanced the oxidative DNA damage induced by 50 mM melamine (Figure 6B), suggesting that the DNA damage may be amplified in the presence of both sodium benzoate and melamine.

Quantitative Evaluation of ROS by DCFA-DA

To determine whether the generation of reactive oxygen species (ROS) may be involved in melamine-induced oxidative DNA damage, the ROS level was evaluated using 2′,7′-dichlorofluorescein diacetate (DCFA-DA). Figure 7A shows a marked and dose-dependent increase in the ROS level in lymphocytes treated with melamine, suggesting that the oxidative DNA damage caused by melamine may be attributable to the generation of ROS. Following treatment with ascorbate and astaxanthin, the ROS level was decreased (Figure 7B), indicating that the suppressive effects of them may result from their radical scavenging activity. The increased ROS level induced by melamine was also reduced to the control level upon treatment

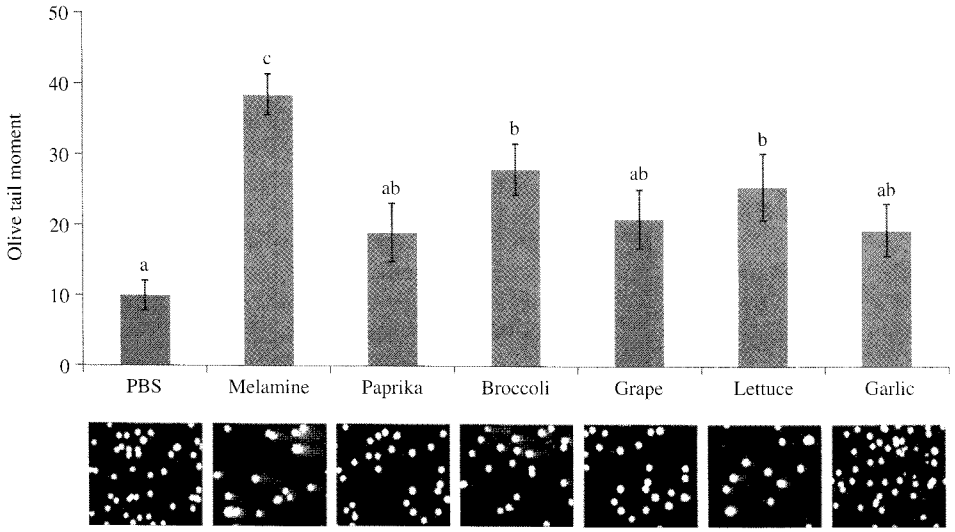

Figure 4. Suppressive effects of edible plant extracts on the melamine-induced oxidative DNA damage in human lymphocytes.

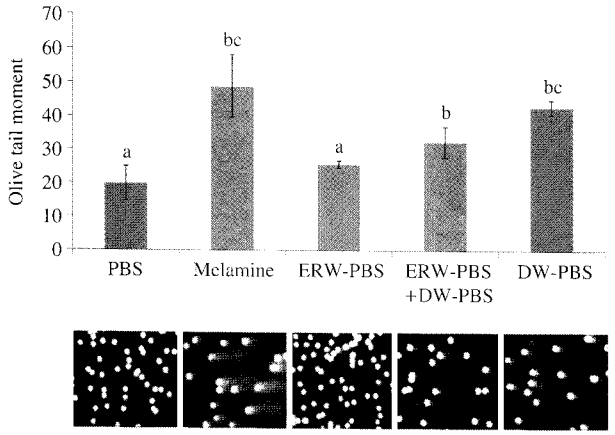

Figure 5. Suppressive effects of electrolyzed-reduced water on the melamine-induced oxidative DNA damage in human lymphocytes.

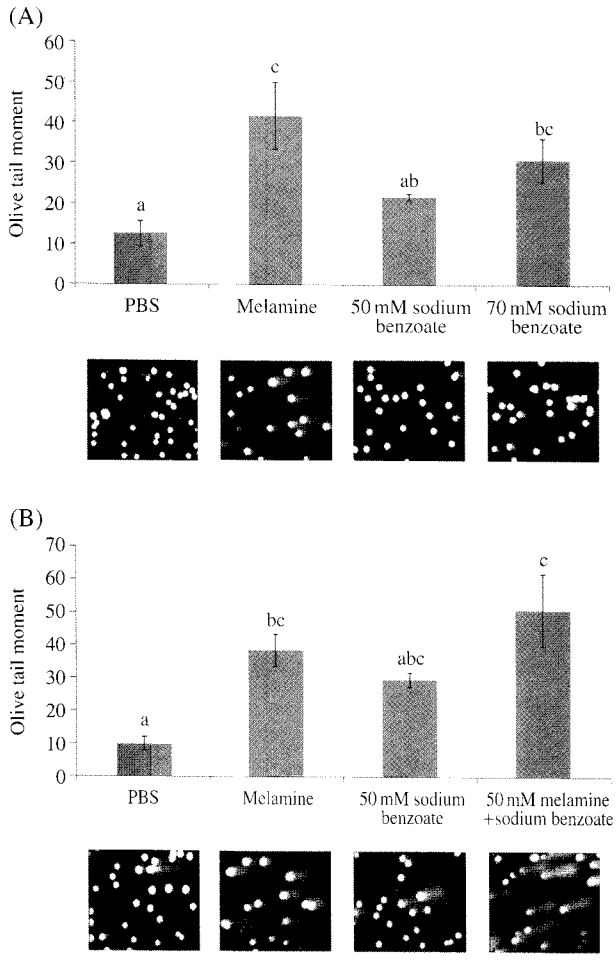

Figure 6. (A) Sodium benzoate-induced oxidative DNA damage in human lymphocytes. (B) Effects of sodium benzoate on the melamine-induced oxidative DNA damage in human lymphocytes.

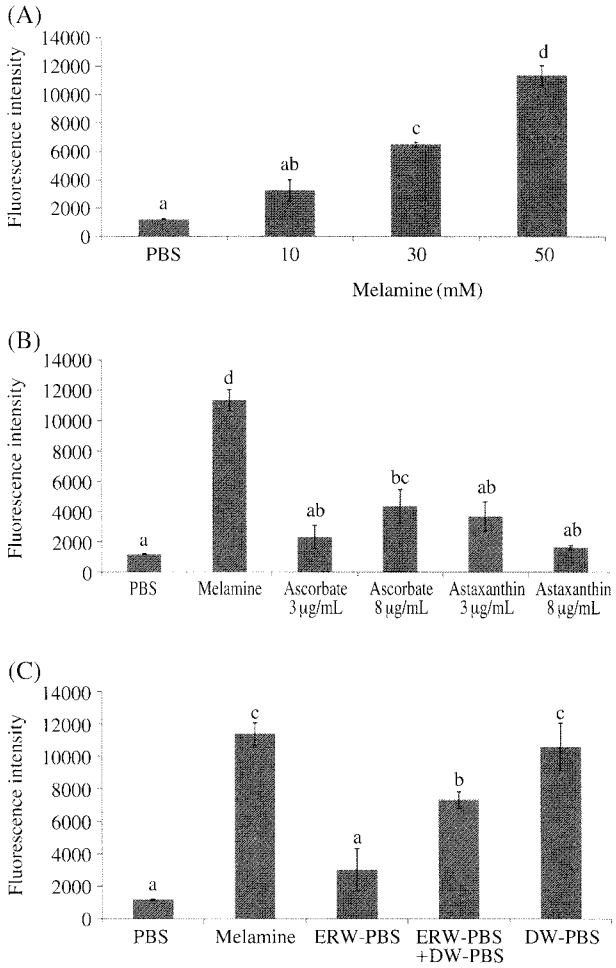

Figure 7. (A) Intracellular reactive oxygen species generation by melamine in human lymphocytes. (B) Suppressive effects of ascorbate and astaxanthin on the reactive oxygen species production in melamine-treated human lymphocytes. (C) Suppressive effects of electrolyzed-reduced water on the reactive oxygen species production in melamine-treated human lymphocytes.

with electrolyzed-reduced water (Figure 7C), and this inhibitory effect decreased when the electrolyzed-reduced water was diluted with deionized water.

Discussion

The results of the present study suggest that melamine induces oxidative DNA damage in human lymphocytes *in vitro* and that various antioxidants inhibit melamine-induced oxidative DNA damage. Melamine alone is not toxic at low doses, and is not classifiable as to its carcinogenicity to humans (Group 3) according to the International Agency for Research on Cancer.

However, experimental studies have shown that the combination of melamine and cyanuric acid leads to crystal formation in renal microtubules and subsequent kidney toxicity[5], and that melamine cyanurate is more toxic than either melamine or cyanuric acid alone. The main toxic effects of dietary exposure to melamine in rats and mice were calculus formation, inflammatory reactions, and hyperplasia in the urinary bladder[17]. Nevertheless, there have been no previous studies of the mechanism of melamine toxicity, including its effects on DNA.

Free radicals and other ROS generated *in vivo* and *in vitro* cause oxidative damage to biomolecules such as DNA, proteins, and lipids[18]. DNA is probably the most biologically significant target of oxidative attack, and it is widely thought that continuous oxidative damage to DNA is a significant contributor to the development of major cancers[19,20]. Antioxidants such as vitamin C, vitamin E (α- and γ-tocopherol), carotenoids (β- and α-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene), and several polyphenolic compounds, including flavonoids (catechins, flavonols, flavones, and isoflavonoids)[21,22], are associated with antioxidative action in biological systems, acting as scavengers of ROS[23,24].

In the present study, the oxidative DNA damage caused by melamine was effectively reduced by several antioxidants and antioxidant-containing Korean medicinal herbs, named *Acanthopanax*, red ginseng and green tea. *Acanthopanax* sp., indigenous to Korea, is a Korean medicinal herb used clinically as a tonic and prophylactic for chronic bronchitis, hypertension, ischemic heart disease, and gastric ulcer, as well as rheumatism, diabetes, and cirrhosis[25]. The major active constituents of the *Acanthopanax* roots are eleutheroside, acanthoside, daucosterine, β-sitosterol, sesamine, and savinine[26]. Ginseng is the root of *Panax ginseng* C.A. Meyer and has been used as a tonic with multiple pharmacological actions *in vitro* and *in vivo*. The ginsenosides, also known as ginseng saponins, are the main molecular components responsible for the action of ginseng, and about 30 different forms have been isolated and identified to date[27-29].

Various edible plant extracts also suppressed melamine-induced DNA damage *in vitro*. Paprika has been reported to have more than twice the amount of ascorbate contained in oranges[30]. Ascorbate has been widely used as an antioxidant to test the suppression of oxidative DNA damage through Comet assay[31]. Broccoli contains many antioxidants, including carotenoids, tocopherols, ascorbate and flavonoids, and has been reported to have high radical scavenging capacity[32]. Grape extract has several biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilatory actions[33], owing to its content of resveratrol, proanthocyanidins, and polymers of catechin and epicatechin. Lettuce is an important source of dietary antioxidants, especially considering its high peroxyl radical scavenging activity[34]. Garlic derivatives have been shown to influence a number of molecular mechanisms in carcinogenesis, including DNA adduct formation, free radicals scavenging, mutagenesis, cell proliferation and differentiation, and angiogenesis[35]. Moreover, a variety of phytochemicals have also been reported to modify the DNA-damaging ability of the alkylating carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine[36]. The activity of 8-oxoguanine DNA glycosylase, a risk factor for lung cancer and head and neck cancer, was decreased by phytochemicals[37].

Electrolyzed-reduced water has recently generated a great deal of interest as a powerful radical scavenger[16]. We showed previously that electrolyzed-reduced water could protect against the oxidative damage of DNA, RNA, and protein[13]. In this investigation, electrolyzed-reduced water also suppressed the oxidative DNA damage caused by melamine.

On the other hand, melamine-induced oxidative DNA damage was enhanced synergistically by the addition of sodium benzoate, compared with the damage produced by either agent alone. The Korea Food and Drug Administration has restricted the legally permitted sodium benzoate level in food to a concentration of 600-1,000 mg/kg. The US Food and Drug Administration has set the limit for sodium benzoate as a preservative at 0.1% by weight.

The results of the present study indicate that melamine might damage human lymphocyte DNA *in vitro* and that antioxidants, acting as radical scavengers, might reduce melamine-induced oxidative DNA damage *in vitro*. Further studies are required to investigate the molecular and cellular mechanism underlying the suppressive effects of various antioxidants on the oxidative DNA damage by melamine.

Materials & Methods

Sample Preparation

Melamine was purchased from Sigma-Aldrich (M2659). Powdered Korean *Acanthopanax* root (Susin Ogapy Co., Ltd., Cheonan-City, Korea) and Korean red ginseng were each dissolved in PBS at a concentration of 3 μg/mL. Polyphenol from green tea was obtained from Hansung Bio Co., Ltd. (Asan, Korea). The electrolyzed-reduced water (ERW) (HDR Co., Ltd. Chuncheon-City, Korea) had a pH of 9 and an oxidation-reduction potential of −150 mV. PBS prepared from ERW alone (ERW-PBS), ERW-PBS diluted

equally with normal PBS (ERW-PBS+DW-PBS), and normal PBS prepared from deionized water alone (DW-PBS) were used for Comet assay.

Paprika, broccoli, grape, lettuce and garlic were obtained from a vegetable and fruit market in Asan, Korea and ground (5 g plant/5 mL PBS) with a food mixer. The extracts were centrifuged at 2,000 rpm for 20 min, and the supernatants were filtered through a Whatman No.1 filter paper. Lymphocytes were treated with each supernatant at a final concentration of 10%. Lymphocytes were incubated with the antioxidants ascorbate and astaxanthin at various concentrations for 30 min at 37°C in the dark and then resuspended in PBS with 50 mM melamine for 5 min on ice. PBS without an oxidative stimulus was used as a negative control.

Determination of DNA Damage by Comet Assay

The alkaline comet assay was performed according to Singh *et al.*[38] with slight modifications. The lymphocytes were mixed with 75 μL of 0.7% low melting point agarose and added to slides precoated with 1.0% normal melting point agarose. After the agarose solidified, the slides were covered with 100 μL of 0.7% low melting point agarose and immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 20 min to allow the DNA to unwind. Electrophoresis was performed at 25 V/300 mA for 20 min at 4°C. The slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C and then treated with ethanol for a further 5 min before staining.

Image Analysis

Slides were stained with ethidium bromide (20 μg/mL) and coverslipped. Measurements were made by image analysis using Komet 5.5 software (Kinetic Imaging, Liverpool, UK) and fluorescence microscopy (Leica, Wetzlar, Germany). To quantify DNA damage in the comet assay, the olive tail moment was calculated as: (Tail.mean-Head.mean) × Tail% DNA/100[39]. A total of 150 randomly captured comets were examined from each slide. The comet slides were codified to ensure the study was performed in a blind manner.

Measurement of Intracellular ROS

The intracellular ROS level was determined by the method of Arai *et al.*[40] with modifications. Aliquots of 20 mL of fresh whole blood were added to 20 mL of PBS and layered onto 40 mL of Histopaque 1077. After centrifugation at 500 × g for 30 min at room temperature, the lymphocytes were collected from just above the Histopaque 1077 boundary and washed twice with 6 mL of PBS. The lymphocytes were incubated with various concentrations of antioxidants and then stimulated with 50 mM melamine for 5 min on ice. Next, aliquots of 7×10^4 cells were incubated with 2 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for an additional 30 min at 37°C in an incubator, and were then washed and resuspended in PBS. The intracellular ROS were detected by fluorescence spectrophotometry (GloMax®-Multi Detection System; Promega, Madison, WI).

Statistical Analysis

The comet assay data are the means of three determinations and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The mean values of DNA damage (olive tail moment) for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. $P < 0.05$ was considered significant.

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