



Inhibitory effect of antioxidants on the benz[a]anthracene-induced oxidative DNA damage in lymphocyte

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Abstract

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Benz[a]anthracene is a ubiquitous environmental contaminant formed during the incomplete combustion of organic material. Some of the metabolites of benz[a]anthracene are known to be toxic and carcinogenic. In this investigation, benz[a]anthracene-induced oxidative damage to lymphocyte DNA was evaluated with the Comet assay (single cell gel electrophoresis). The level of oxidative DNA damage caused by benz[a]anthracene increased in a dose-dependent manner (24, 49) and oxidative DNA damage was significantly inhibited by 5 and 10 $\mu\text{g ml}^{-1}$ ascorbate, 5 $\mu\text{g ml}^{-1}$ polyphenols, as well as 5 and 10 $\mu\text{g ml}^{-1}$ curcumin. Moreover, traditional Korean medicinal herbs such as Acanthopanax and ginseng significantly reduced DNA damage. The results demonstrate that antioxidant supplementation to lymphocytes inhibits oxidative DNA damage *in vitro*, supporting an inhibitory effect against oxidative DNA damage, probably due to reduction of reactive oxygen species production induced by benz[a]anthracene.

Key words

Benz[a]anthracene, Comet assay, Oxidative DNA damage, Antioxidant

Introduction

Benz[a]anthracene is a ubiquitous contaminant derived from the incomplete combustion of organic material, along with a number of other polycyclic aromatic hydrocarbons (Slaga *et al.*, 1979; Rana and Verma, 2005). The possibility of health hazards resulting from exposure to benz[a]anthracene has been extensively studied, and experimental and epidemiological data suggest that neurotoxic (Hiemstra *et al.*, 2007), hepatotoxic (Muller *et al.*, 2007), and nephrotoxic (Tatu *et al.*, 1998) side effects can arise from benz[a]anthracene exposure. The arrangement of the aromatic rings in the benz[a]anthracene molecule gives it a "bay region," which is often correlated with carcinogenic properties. Thus, some of the metabolites are known to react with cellular macromolecules, including DNA, which may account for both the toxicity and carcinogenicity (Yu *et al.*, 2006). The inducible microsomal enzyme, cytochrome P-450 mixed-function oxidase system, might oxidize benz[a]anthracene to excretable metabolites with increased water solubility. Unfortunately, a minor product of this oxidation, a bay-region diol epoxide, can readily react with DNA and greatly increase carcinogenic activity (Slaga *et al.*, 1979). Benz[a]anthracene has

been classified as probably carcinogenic to humans, in Group 2B according to the International Agency for Research on Cancer (IARC, 2010). No commercial application for benz[a]anthracene exists; however, it is found in various products used in everyday life: smoke, flue gases, charcoal, barbecued meats, coal tar, petroleum asphalt, and a variety of foods, including vegetable oils and baker's yeast.

The single-cell gel electrophoresis assay, known as the Comet assay, is a well-established genotoxicity test for detection of DNA damage at the level of individual eukaryotic cells both *in vivo* and *in vitro* (Park *et al.*, 2005). It has been widely used to detect primary DNA damage in human and animal cells exposed to various environmental or occupational toxicants (Garaj-Vrhovac *et al.*, 2009; Lee *et al.*, 2007a,b). 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 development of major cancers (Totter, 1980; Ames *et al.*, 1993). Free radicals and other reactive oxygen species (ROS) generated *in vivo* and *in vitro* cause oxidative damage to biomolecules such as DNA (Grune and Davies, 1997).

A variety of antioxidants and medicinal herbs have been known to suppress the generation of reactive oxygen species. Curcumin is the principal curcuminoid of the popular Indian spice turmeric (Choi, 2009) and a wide range of potential therapeutic or preventive effects have been suggested to be associated with curcumin (Wongcharoen *et al.*, 2009). It has antioxidant, anti-inflammatory, antiviral, and antifungal actions, as well as an anticancer effect (Anand *et al.*, 2008). Curcumin can interfere with the transcription factor NF- κ B, which has been linked to a number of inflammatory diseases and cancer (Biswas and Rahman, 2008), and can bind heavy metals, thereby decreasing the toxicity of cadmium and lead (Rolli *et al.*, 2010).

Acanthopanax sp. is used clinically as a tonic and prophylactic for chronic bronchitis, hypertension, ischemic heart disease, gastric ulcer, rheumatism, diabetes, and cirrhosis (Yi *et al.*, 2001; Park *et al.*, 2007). The major active constituents of *Acanthopanax* are eleutheroside, acanthoside, daucosterine, β -sitosterol, sesamine, and savinine (Shan *et al.*, 1999). Ginseng (*Panax ginseng* C.A. Meyer), indigenous to Korea, has multiple known pharmacological efficacies (Hong *et al.*, 2002). Ginsenosides are saponins and are the main molecular component responsible for the pharmacological effects of ginseng (Kim *et al.*, 2009), with more than 30 different types found to date (Lee *et al.*, 2007; Li *et al.*, 2007; Ko *et al.*, 2007).

Much information on the toxicity and carcinogenic effects of benz[a]anthracene is already available. However, information on oxidative DNA damage and prevention using antioxidants evaluated has been very limited (Park and Lee, 2009). In this study, benz[a]anthracene-induced DNA damage to lymphocytes was examined with the Comet assay. The suppressive effects of various antioxidants, including Korean medicinal herbs, on the oxidative DNA damage produced by benz[a]anthracene were also investigated.

Materials and Methods

Preparation of lymphocytes: A 200 μ l of fresh whole blood from a healthy volunteer was added to 800 μ l of phosphate-buffered saline (PBS) and layered onto 200 μ l of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,450 rpm for 5 min at room temperature, the lymphocytes were collected from the layer just above the Histopaque 1077 boundary and washed in 1 ml PBS.

Treatment of lymphocytes: Powdered whole (3 μ g ml⁻¹) red ginseng (*Panax ginseng* C.A. Meyer), *Acanthopanax* (3 μ g ml⁻¹) (*Acanthopanax sp.*) and polyphenols (1,3 and 5 μ g ml⁻¹) from green tea leaves were each dissolved in PBS. Curcumin was (5 and 10 μ g ml⁻¹) dissolved in DMSO and then diluted with PBS. To investigate the ability of antioxidants to inhibit oxidative DNA damage, lymphocytes were pre-incubated with various concentrations of the above antioxidants for 30 min at 37°C in the dark, and then treated with 10 μ M benz[a]anthracene for 5 min on ice for pre-treatment of lymphocytes with antioxidants. In case of post-treatment

of lymphocytes with antioxidants, lymphocytes were incubated with curcumin for 30 min at 37°C after 10 μ M benz[a]anthracene treatment for 5 min on ice. PBS treated sample was used as a negative control.

Determination of DNA damage by the Comet assay: The alkaline Comet assay was performed according to Singh *et al.* (1988) with slight modifications, as in our earlier reports (Park *et al.*, 2005). The lymphocytes were mixed with 75 μ l 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 μ l 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 hr at 4°C. The slides were placed in an electrophoresis tank containing 300 mM NaOH and 10 mM Na₂EDTA (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-HCl, pH 7.5) for 5 min at 4°C and then treated with ethanol for 5 min. Slides were stained with ethidium bromide (20 μ g ml⁻¹) and measured using a fluorescence microscope (Leica, Wetzlar, Germany), with image analysis using Komet 5.5 software (Kinetic Imaging, Liverpool, UK). To quantify DNA damage in the Comet assay, the olive tail moment was calculated as (Tail.mean-Head.mean) \times Tail% DNA/100 (Sul *et al.*, 2008). 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.

Statistical analysis: The Comet assay data are 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.

Results and Discussion

Lymphocytes treated with 5–50 μ M benz[a]anthracene exhibited oxidative DNA damage, as determined by the olive tail moment in a Comet assay (Fig. 1). The olive tail moment at 10 μ M benz[a]anthracene was about 24.49 ± 1.62 compared with 7.13 ± 1.62 in the PBS-treated control, indicating an approximately three-fold increase in DNA damage. Hydrogen peroxide is generally used to detect DNA strand breakage by generating hydroxyl radicals close to the DNA molecule in the Comet assay (Diplock, 1991), and 150 μ M H₂O₂ showed a tail %DNA of about 30, which is an approximately three- to four-fold increase in DNA damage compared with the control (Cemeli *et al.*, 2003). These results indicate that benz[a]anthracene-induced oxidative DNA damage is more severe than that induced by H₂O₂ (Hartwing, 2002).

The addition of ascorbate inhibited the oxidative DNA damage caused by 10 μ M benz[a]anthracene (Singh and Rana, 2007), as demonstrated by a reduction in the olive tail moment in a Comet assay after ascorbate treatment (Fig. 2). The effect of

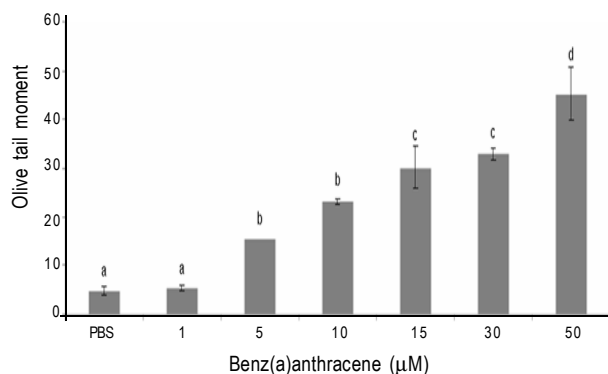


Fig. 1: DNA damage in lymphocytes induced by different concentrations of benz[a]anthracene (1-50 μM). Values not sharing the same letter are significantly different from one another (p<0.05)

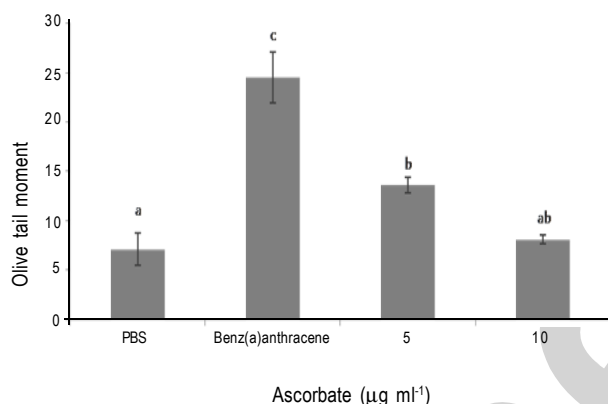


Fig. 2: Effect of ascorbate (5 and 10 μg ml⁻¹) on the benz[a]anthracene (10 μM)-induced oxidative DNA damage in lymphocytes. Values not sharing the same letter are significantly different from one another (p<0.05)

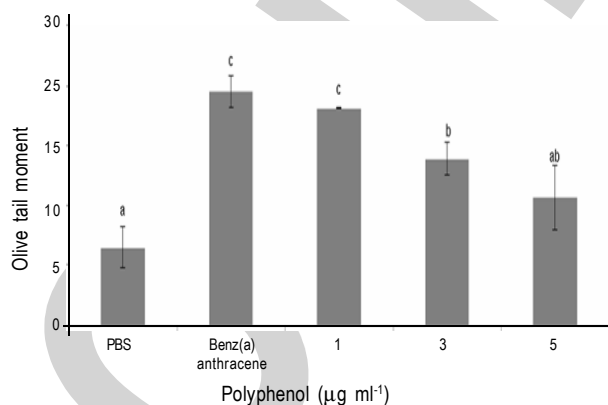


Fig. 3: Effect of polyphenols (1, 3 and 5 μg ml⁻¹) on benz[a]anthracene (10 μM)-induced DNA damage in lymphocytes. Values not sharing the same letter are significantly different from one another (p<0.05)

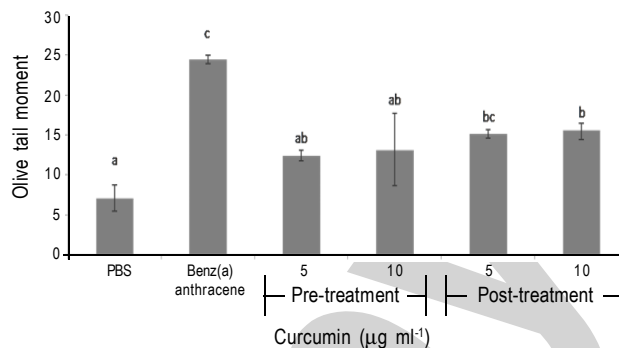


Fig. 4: Effect of pre-treatment and post-treatment with curcumin (5 and 10 μg ml⁻¹) on benz[a]anthracene (10 μM)-induced DNA damage in lymphocytes. Values not sharing the same letter are significantly different from one another (p<0.05)

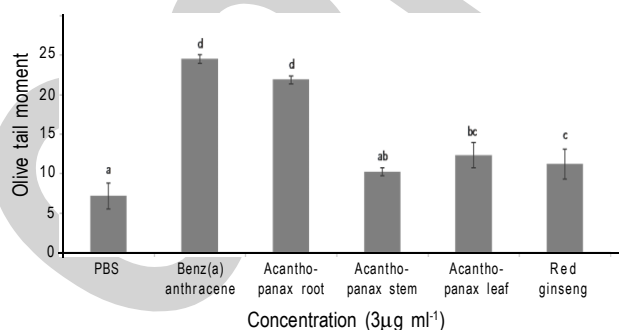


Fig. 5: Effects of the traditional Korean medicinal herbs *Acanthopanax* (3 μg ml⁻¹) and ginseng (3 μg ml⁻¹) on benz[a]anthracene (10 μM)-induced DNA damage in lymphocytes. Values not sharing the same letter are significantly different from one another (p<0.05)

polyphenols from Korean green tea leaves was also tested (Fig. 3), revealing a significant reduction in the olive tail moment in lymphocytes treated with benz[a]anthracene. Fig. 4 shows the effects of curcumin on benz[a]anthracene-induced DNA damage *in vitro*. When curcumin was preincubated with lymphocytes before benz[a]anthracene treatment, a notable reduction in the olive tail moment was observed, suggesting the preventive effect of curcumin on the benz[a]anthracene-induced oxidative DNA damage. Adding curcumin following benz[a]anthracene treatment to cause DNA damage also led to a reduction in the olive tail moment. The results indicate that both pre-treatment and post-treatment with curcumin seem to be efficacious against benz[a]anthracene-induced DNA damage.

Fig. 5 shows the herbs *Acanthopanax* and ginseng could suppress benz[a]anthracene-induced DNA damage *in vitro*. Phytochemicals, including polyphenols, carotenoids, and flavonoids, are known to be powerful natural antioxidants found in many Korean medicinal plants. Moreover, they have antioxidative action in biological systems, acting as scavengers of ROS (Rice-Evans *et al.*, 1995; Jorgensen *et al.*, 1999). Thus, the antioxidative activities of these herbs through scavenging ROS may account for their

suppressive effects on oxidative DNA damage after benz[a]anthracene treatment.

The present study showed that benz[a]anthracene damages human lymphocyte DNA *in vitro* and that antioxidants, probably acting as radical scavengers, inhibit benz[a]anthracene-induced oxidative DNA damage *in vitro*. Further studies are required to determine the molecular and cellular mechanisms underlying the suppressive effects of these antioxidants on benz[a]anthracene-induced oxidative DNA damage.

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