



## Anti-oxidative and anti-inflammatory activities of placental extracts in benzo[a]pyrene-exposed rats

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### ABSTRACT

**Objective:** Placental extracts (PE) have been used for years as a folk remedy in Asian countries. PE mediates alleviation of menopausal symptoms, wound healing, liver regeneration and anti-inflammatory responses. In this study, we evaluated the protective effects of PE on rats exposed to benzo[a]pyrene (BaP).

**Methods:** The composition of amino acids, sugars and fatty acids in PE was analyzed. The effect of PE on DNA damage was determined by Comet assay, and oxidative damage was determined by measuring the activity of superoxide dismutase and the levels of lipid peroxidation. The effect of PE on cytokines and immunoglobulin levels was determined by western blot analysis.

**Results:** Exposure of rats to BaP significantly increased the Olive Tailmoments compared to controls, while pre-treatment with PE composed of diverse amino acids, monosaccharides and fatty acids significantly decreased the Olive Tailmoments induced by BaP. In addition, oxidative stress induced by BaP was attenuated by pre-treatment with PE. Furthermore, PE pre-treatment significantly decreased the levels of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6.

**Conclusion:** Pre-treatment of rats with PE significantly attenuates oxidative damage and immunotoxicity induced by BaP. These findings suggest the further studies regarding the protective effects of PE against environmental toxicants in humans.

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### 1. Introduction

Placental extracts (PE) have been used for years as a wound healer and a cosmetic in many countries including India [1]. It is enriched in enzymes, nucleic acids, vitamins, amino acids, steroids, fatty acids, and minerals [2]. PE mediates immunotrophic, anti-oxidative, and anti-inflammatory responses. The major anti-oxidant components in PE are uracil, tyrosine, phenylalanine, and tryptophan. About 59% of the anti-oxidative effects of PE can be attributed to these components [3]. Peptides produced from collagen have also been reported to be anti-oxidative components of PE [4]. PE is widely marketed in Asian countries for its wound healing, immunotrophic and anti-inflammatory activities [2].

Benzo[a]pyrene (BaP) is a well-known carcinogen classified into a Group 1 polycyclic aromatic hydrocarbon (PAH) by the

International Agency for Research on Cancer (IARC) [5]. It is present in food, the workplace, and the environment. Grilled or smoked meat is the major source of dietary BaP intake [5]. BaP undergoes a metabolic activation to form reactive intermediates such as BaP-quinones. These intermediates are known to be involved in generating reactive oxygen species (ROS) and are associated with oxidative alteration of DNA, proteins, and anti-oxidant enzymes [6]. These reactive intermediates have mutagenic and carcinogenic effects in biological systems [7].

In the present study, we evaluated the protective effects of PE in rats exposed to BaP. To determine if PE protects lymphocytes from BaP-induced DNA damage, rats were exposed to PE-only, BaP-only or BaP + PE, and lymphocytes were then separated from the whole blood of rats and assayed using a Comet assay. We also measured levels of superoxide dismutase (SOD), malondialdehyde (MDA), and carbonyl to evaluate the possible anti-oxidant effect of PE against BaP in rat plasma. Furthermore, we evaluated the anti-inflammatory effect of PE treatment by examining the levels of immunoglobulins and pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6.

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## 2. Materials and methods

### 2.1. Chemicals

BaP, benzyl alcohol, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human PE was provided by Melsmon Pharmaceutical Co., Ltd. (Tokyo, Japan). PE (2 ml) contains 100 mg of constituents and 0.03 ml of benzyl alcohol.

### 2.2. High performance liquid chromatography (HPLC) analysis of amino acids

The HPLC equipment used in the present study was the Shimadzu System (Shimadzu, Kyoto, Japan), which included Shimadzu LC-10ADVP pumps and a Shimadzu RF-10AXL fluorescence detector. Shim-pack Amino–Na columns (6.0 mm × 100 mm) were used with guard columns (Shim-pack ISC-30Na, Shimadzu). The column temperature was set at 60 °C by using a Shimadzu CTO-10ACVP column oven. The mobile phase was Mobile Phase Kit–Na, gradient elution (A: sodium citrate buffer, B: sodium citrate buffer containing boric acid, C: sodium hydroxide solution). The mobile phase was filtered under vacuum through a 0.45 µm membrane filter and degassed before use. The analysis was carried out at a flow rate of 0.4–0.6 ml/min with the detection wavelength set at excitation 254 nm and emission 450 nm, and the injection volume set at 20 µl. The mixture of amino acids type H used as the standard was diluted to 0.1 mol/ml in sodium citrate solution. Fifty microliters of PE was diluted 20 times with the sodium citrate solution for the HPLC analysis.

### 2.3. Monosaccharide composition analysis

The amount of monosaccharides was measured by using a Bio-LC (DX-300, Dionex Corp., Sunnyvale, CA, USA) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex) with a guard column (4 × 50 mm) followed by the method published elsewhere [8]. Monosaccharides were separated with 16 mM NaOH at a flow rate of 1 ml/min.

### 2.4. Lipids extraction and analysis of fatty acids by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS)

Total lipids were extracted using the monophasic neutral organic solvent system containing 0.01% butylated hydroxytoluene [9]. Fatty acid methyl esters (FAME) were prepared by heating at 60 °C for 30 min with boron trifluoride (BF<sub>3</sub>, Sigma Chemical Co.). FAME were analyzed by GC using a Hewlett–Packard 6890N series gas–liquid chromatography (Agilent Technologies, Wilmington, NC, USA) equipped with a capillary column (30 m × 0.25 mm) coated with a film of DB-23 (J & W Scientific, Folsom, CA, USA). FAME were dissolved in hexane, injected onto the column, and separated by employing a temperature program from 150 to 250 °C at 2 °C/min, with both the injector and the flame ionization detector temperature set at 250 °C. Helium (ultra pure grade) was used as the carrier gas at a flow rate of 1 ml/min and a split ratio of 50:1 ratio mode. The gas chromatographic identification of fatty acids was verified by GC/MS on an Agilent 6890 GC equipped with an HP-5 column (Agilent Technology). The GC was interfaced to a high-resolution Agilent 5973 mass spectrometer. The obtained mass spectral data were analyzed using an Agilent G1034 mass spectrometry chemstation. Fatty acids were identified according to their elemental composition, the mass of their molecular ions, and the fragmentation patterns compared to those obtained from authentic standards.

### 2.5. Experimental design

Male Sprague–Dawley rats (173 ± 5 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). They were housed under standard laboratory conditions (Temperature 24 ± 2 °C; humidity 50 ± 10%, 12-h day/night cycles). Animals were allowed to acclimatize to the facility for 1 week, and were provided standard chow diet and drinking water *ad libitum*. Eighty rats (6 weeks old, 5 per group) were divided among the following four groups: 1) a control group (vehicle only), 2) a PE exposure group (20 µl × 3 times/week for 2 weeks, intramuscular injection), 3) a BaP exposure group (200 mg/kg body weight, intraperitoneal injection) and 4) a PE group (20 µl × 3 times/week for 2 weeks, intramuscular injection followed by BaP at 200 mg/kg body weight, intraperitoneal injection). The rats in the non-PE-treated groups and PE-treated groups were injected with either diluted benzyl alcohol 20 µl (0.03 ml of benzyl alcohol diluted in 2 ml of a saline solution) or 20 µl of PE, respectively. After 2 weeks of injections, BaP in corn oil or corn oil only was injected into the BaP-treated groups and the BaP-untreated groups, respectively. Rats were then sacrificed 1, 2, 4, or 8 days after BaP injection according to the toxicokinetic results of BaP [10].

### 2.6. Blood sample preparation

Blood samples (EDTA-treated whole blood) were collected from each rat by cardiac puncture. For the Comet assay, lymphocytes were prepared by removing red blood cells from the whole blood (3–5 ml) via centrifugation with a Ficoll-Plaque

solution. For other experiments, whole blood was centrifuged at 4000 rpm at 4 °C for 20 min and the resulting plasma was aliquoted and stored at –70 °C until use.

### 2.7. Comet assay

The comet assays were performed as described previously [11]. For each treatment group, two slides were prepared and each 50–100 randomly chosen cells (total 100–200 cells) were scored manually. The parameter, Olive Tailmoment [(Tail.mean – Head.mean) × Tail%DNA/100], was automatically calculated using the Komet 4.0 image analysis system (Kinetic Imaging; Liverpool, UK).

### 2.8. Determination of oxidative damage

The activity of the anti-oxidant enzyme SOD was measured according to the instructions provided with the kit (Cayman Chemical Company; Ann Arbor, MI, USA). MDA was measured using a thiobarbituric acid test and an HPLC method [12]. The standard curve was made from 1,1,3,3-tetraethoxypropane (TEP) because the acidic hydrolysis of TEP yields stoichiometric amounts of MDA [13].

The level of protein oxidation was evaluated by western blot analysis using anti-dinitrophenylhydrazine antibody. Ten µg of proteins was separated electrophoretically and transferred onto polyvinylidene difluoride (PVDF) membrane. Thereafter, the membrane was incubated for 1 h with anti-dinitrophenylhydrazine antibody (1:4000; Invitrogen; Carlsbad, CA, USA), and developed with enhanced chemiluminescence reagents (Amersham Biosciences; Piscataway, NJ, USA). Densitometry was then performed using a Bio-Rad (Hercules, CA, USA) 700 flatbed scanner and Molecular Analyst software (Bio-Rad).

### 2.9. Protein determination, electrophoresis, and immunoblotting

The plasma was mixed with Laemmli buffer and boiled in a boiling water bath for 10 min. The proteins were separated electrophoretically and transferred to PVDF membranes. The following antibodies were used for immunodetection: horseradish peroxidase (HRP) conjugated goat anti-rat IgG1 (1 µg/ml; Immunology Consultants Laboratory, Newberg, OR, USA), HRP conjugated goat anti-rat IgG2a, goat anti-rat TNF-α (1 µg/ml; R&D systems, Minneapolis, MN, USA), goat anti-rat IL-1β (1 µg/ml; R&D systems), goat anti-rat IL-6 (1 µg/ml; R&D systems), goat anti-mouse α-tubulin (clone DM1A; 1:20,000; Sigma–Aldrich). Goat anti-rat and goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:1000; Promega; Madison, WI, USA) followed by enhanced chemiluminescence reagents (Amersham Biosciences) were used to detect the target proteins. Immunoreactive bands were imaged using a ChemiDoc XRS system (Bio-Rad). Densitometry of these bands was performed using Quantity One software (Bio-Rad) and values were normalized using α-tubulin as an internal loading control.

### 2.10. Data analysis

Data in text and figures are expressed as mean ± SD. Two-group comparisons were evaluated with one-way ANOVA test. Differences were considered statistically significant at *p* < 0.05.

## 3. Results

### 3.1. Placental extract contains diverse amino acids, sugars and lipids

#### 3.1.1. Amino acids

The quantities of amino acids in PE were calculated based on the HPLC analysis data. The primary amino acids eluted sequentially from the Shim-pack Amino–Na columns reacted with the continuously added OPA reagent (*o*-phthalaldehyde), and were converted to fluorescent derivatives. As shown in Table 1, glutamic acid,

**Table 1**  
Amino acid contents in placental extracts.

Amino acids	Contents (mg/ml)	Amino acids	Contents (mg/ml)
Aspartic acid	0.495 ± 0.008	Methionine	0.055 ± 0.002
Threonine	0.232 ± 0.004	Isoleucine	0.130 ± 0.001
Serine	0.283 ± 0.007	Leucine	0.483 ± 0.014
Glutamic acid	0.965 ± 0.017	Tyrosine	0.133 ± 0.005
Proline	0.275 ± 0.005	Phenylalanine	0.142 ± 0.004
Glycine	0.468 ± 0.007	Histidine	0.057 ± 0.002
Alanine	0.362 ± 0.009	Lysine	0.312 ± 0.004
Valine	0.205 ± 0.008	Arginine	0.370 ± 0.010

**Table 2**  
Sugar contents in placental extracts.

Sugars	Contents (g/100 g)
Glucose	0.01 ± 0.000
Galactosamine	0.05 ± 0.015
Glucosamine	0.10 ± 0.012
N-acetylneuraminic acid	0.23 ± 0.029
N-glycolyneuraminic acid	0.02 ± 0.006

glycine and aspartic acids were the major amino acids included in PE, whereas tyrosine and phenylalanine were also detected with concentrations of  $0.133 \pm 0.005$ ,  $0.142 \pm 0.004$  mg/ml, respectively.

### 3.1.2. Monosaccharides

Sugars in PE were separated and detected by using a Bio-LC DX-300 system equipped with a CarboPac PA1 column. N-Acetylneuraminic acid, an N-acyl derivative of amino sugar derivatives, was detected to have the largest amount in PE. N-glycolyneuraminic acid, glucosamine, galactosamine, and glucose were also detected in PE (Table 2).

### 3.1.3. Fatty acids

The major saturated fatty acid of the total extractable lipids in PE was 18:0 with smaller amounts of 16:0, while 18:1 (9) was the largest unsaturated fatty acids present in PE (Table 3). 16:1 (9) and 18:1 (9) were the monoenoic monounsaturated fatty acids, and 18:2 (9,12), 20:4 (5,8,11,14), and 22:6 (4,7,10,13,16,19) were polyunsaturated fatty acids found in PE. Among them, 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19) were highly unsaturated fatty acids of the omega-3 type in PE. Total SFA occupied  $78.51 \pm 1.240\%$ , whereas total USFA occupied  $8.95 \pm 0.686\%$  of total extractable lipids in PE. The ratio of SFA/USFA was 8.77.

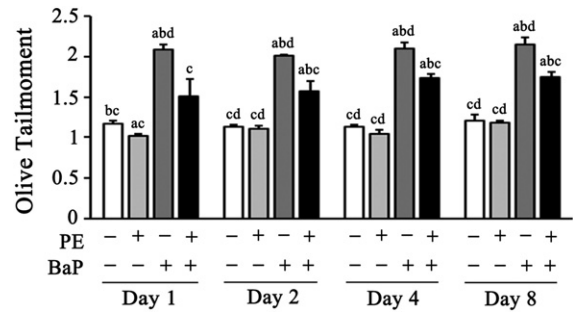
### 3.2. Placental extract attenuates DNA damage induced by BaP in rats

To evaluate the protective effect of PE against DNA damage induced by BaP in rats, lymphocytes prepared from whole blood were subjected to the Comet assay. The Olive Tailmoments of PE-treated rats in the 1-day group were decreased compared to control groups, whereas those in the 2-, 4-, and 8-day groups were not significantly different from control groups (Fig. 1). In contrast, exposure to BaP significantly increased the Olive Tailmoments

**Table 3**  
Fatty acid composition from total extractable lipids of placental extracts.

Fatty acid	RRT	Relative amount (%)
14:0	0.333	8.24 ± 3.510
16:0	0.549	11.77 ± 1.392
16:1 (9)	0.601	0.69 ± 0.277
18:0	1.000	54.47 ± 2.018
18:1 (9)	1.086	3.27 ± 2.079
18:2 (9,12)	1.224	0.68 ± 0.113
20:0	1.452	0.64 ± 0.009
20:1 (9)	1.487	1.30 ± 0.566
20:4 (5,8,11,14)	1.652	1.53 ± 0.662
22:0	1.858	1.50 ± 0.619
22:6 (4,7,10,13,16,19)	2.450	1.48 ± 0.615
26:0	2.883	1.24 ± 0.552
28:0	3.443	0.64 ± 0.196
Other <sup>a</sup>		12.54 ± 0.931
SFA		78.51 ± 1.240
USFA		8.95 ± 0.686
SFA/USFA		8.77

<sup>a</sup> Unidentified fatty acids.



**Fig. 1.** Protective effect of placental extracts (PE) on DNA damage induced by BaP in rats. The effect of PE on DNA damage induced by benzo[a]pyrene (BaP) was evaluated using a Comet assay to measure single-strand DNA breaks at the single-cell level. BaP treatment increased the Olive Tailmoments above control levels, while pre-treatment with PE prior to BaP exposure significantly decreased the Olive Tailmoments compared to BaP treatment alone. All data represent the mean ± SD of five different rats. <sup>a</sup>*p* < 0.05 compared to the control rats, <sup>b</sup>*p* < 0.05 compared to PE-treated rats, <sup>c</sup>*p* < 0.05 compared to BaP-treated rats, <sup>d</sup>*p* < 0.05 compared to PE + BaP-treated rats.

compared to control rats and PE-treated rats in all groups. Conversely, rats pre-treated with PE for 2 weeks before BaP exposure exhibited a significant decrease in the Olive Tailmoments in all groups compared to BaP-only treated rats.

### 3.3. Placental extract attenuates BaP-induced oxidative damage in rats

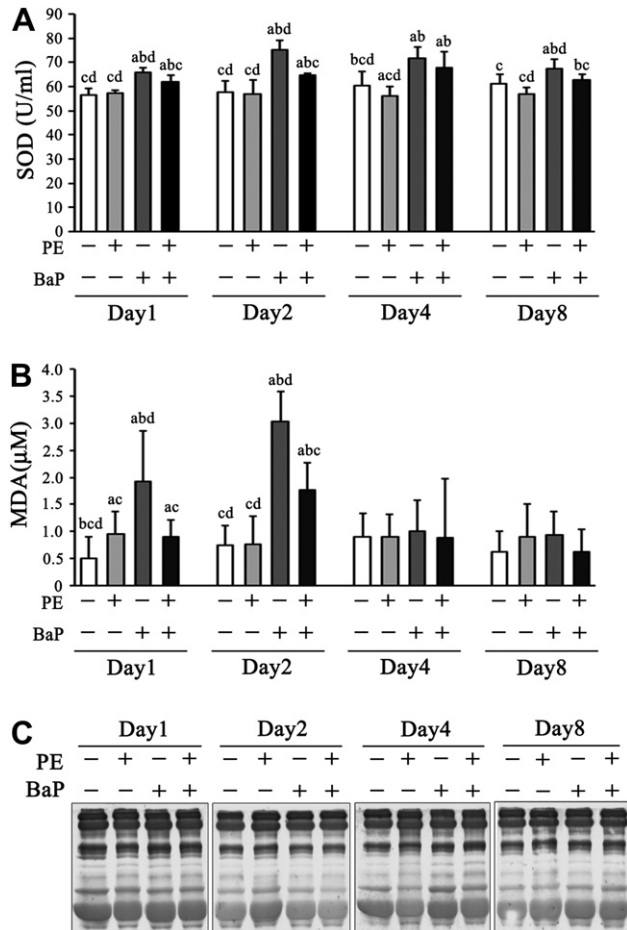
SOD, lipid peroxidation, and protein oxidation were evaluated to determine if PE has an anti-oxidative protective effect. The levels of SOD in PE-only treated rats were not significantly different from those in controls. However, the levels of SOD in BaP-exposed rats in the 1-, 2-, 4-, and 8-day groups were significantly increased compared to controls and PE-only treated rats (Fig. 2A). Conversely, exposure to PE prior to BaP significantly attenuated the activity of SOD in the 1-, 2-, and 8-day groups compared to BaP-only treated rats.

The anti-oxidant effect of PE was also evaluated by measuring the amount of MDA produced in rat plasma using a thiobarbituric acid test. As shown in Fig. 2B, the production of MDA in control rats was not significantly different among the 1-, 2-, 4-, and 8-day groups. Treatment with PE alone did not increase the production of MDA, either. However, exposure to BaP in the 1- and 2-day groups significantly increased the levels of MDA compared to the control rats, whereas exposure of rats in the 4- and 8-day groups to BaP did not affect the production of MDA compared to the control rats. Conversely, pre-treatment with PE significantly decreased the levels of MDA in the 1- and 2-day groups compared to BaP-only treated rats.

Next, we evaluated the effect of PE on protein oxidation induced by BaP (Fig. 2C) by western blot analysis using anti-dinitrophenylhydrazine antibody. The level of immunoreactivity to the anti-dinitrophenylhydrazine antibody did not change significantly in any of the groups compared to the control, regardless of whether the rats were treated with PE or exposed to BaP.

### 3.4. Placental extract does not increase the levels of immunoglobulins in rats exposed to BaP

The levels of the two most abundant immunoglobulins in the plasma, IgG1 and IgG2a, were measured in rat plasma by western blot analysis (Fig. 3). Treatment with PE alone significantly increased the levels of IgG1 only in the 2-day group, whereas the immune boosting effect of PE was not observed in other groups of

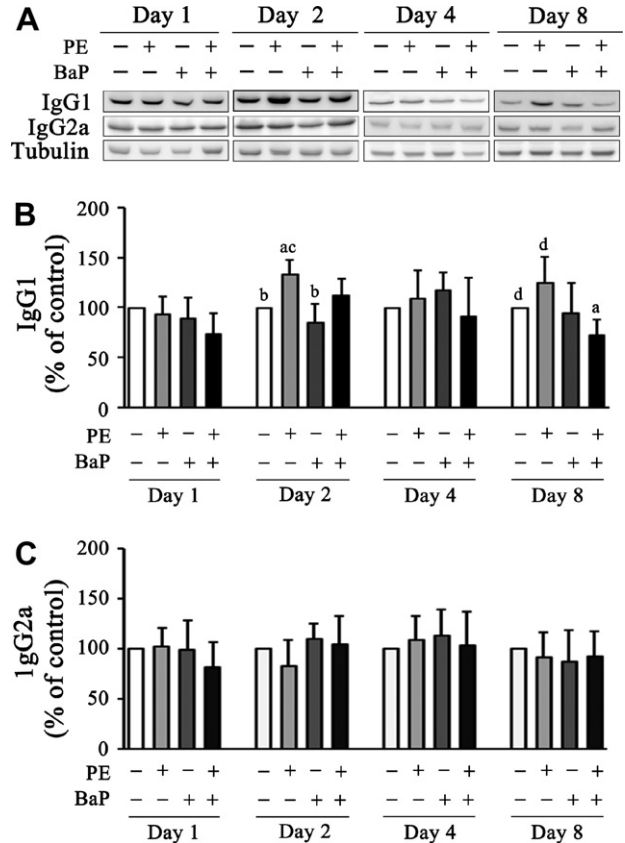


**Fig. 2.** The anti-oxidant effect of PE against BaP-induced oxidative stress. Plasma collected from rats exposed to BaP with or without PE pre-treatment was evaluated for oxidative stress markers. (A) The detoxification enzyme, SOD, was measured using commercially available kits. The BaP-induced elevation of SOD was significantly decreased by pre-treatment with PE in the 1-, 2-, and 8-day groups. (B) The levels of lipid peroxidation were evaluated by measuring the amounts of malondialdehyde using a high performance liquid chromatography method. (C) The levels of protein oxidation were evaluated by western blot analysis using an anti-dinitrophenylhydrazine antibody. All data represent the mean  $\pm$  SD of five different rats. <sup>a</sup> $p < 0.05$  compared to the control rats, <sup>b</sup> $p < 0.05$  compared to PE-treated rats, <sup>c</sup> $p < 0.05$  compared to BaP-treated rats, <sup>d</sup> $p < 0.05$  compared to PE + BaP-treated rats.

rats pre-treated with PE. However, exposure to BaP did not significantly affect the level of IgG1. Pre-treatment with PE before exposure to BaP did not have a significant effect on the levels of IgG1. In addition, the level of IgG2a was not significantly different among the control, PE-only treated, BaP-only treated, and PE + BaP-treated rats.

### 3.5. PE attenuate inflammation induced by BaP in rats

The effects of PE on the levels of BaP-induced pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, were examined by western blot analysis (Fig. 4). Treatment of rats with PE-only did not increase the expression levels of TNF- $\alpha$  in any of the groups. BaP exposure did not significantly increase the levels of TNF- $\beta$  compared to control rats in the 1-day group, either. However, in the 2-, 4-, and 8-day groups, BaP exposure significantly increased the levels of TNF- $\alpha$  compared to control rats. The increased levels of TNF- $\alpha$  in rats in the 2- and 4-day groups were not attenuated by pre-treatment with PE prior to BaP. The only significant decrease in TNF- $\alpha$  levels



**Fig. 3.** The effect of placental extracts (PE) on immunoglobulin levels in rats exposed to benzo[a]pyrene (BaP). (A) Western blot analysis of immunoglobulins in plasma collected from rats exposed to BaP for 1, 2, 4, or 8 days with or without PE pre-treatment using anti-IgG1 and IgG2a antibodies. An equal amount of total protein was loaded into each lane. (B, C) Graphs showing changes in the levels of IgG1 and IgG2a in plasma from rats exposed to BaP with or without PE. Densitometric values were normalized using  $\alpha$ -tubulin as an internal control. Values are expressed as a percentage of control rats in each group. All data represent the mean  $\pm$  SD of five different rats. <sup>a</sup> $p < 0.05$  compared to the control rats, <sup>b</sup> $p < 0.05$  compared to PE-treated rats, <sup>c</sup> $p < 0.05$  compared to BaP-treated rats, <sup>d</sup> $p < 0.05$  compared to PE + BaP-treated rats.

occurred in the 8-day rats pre-treated with PE prior to BaP exposure.

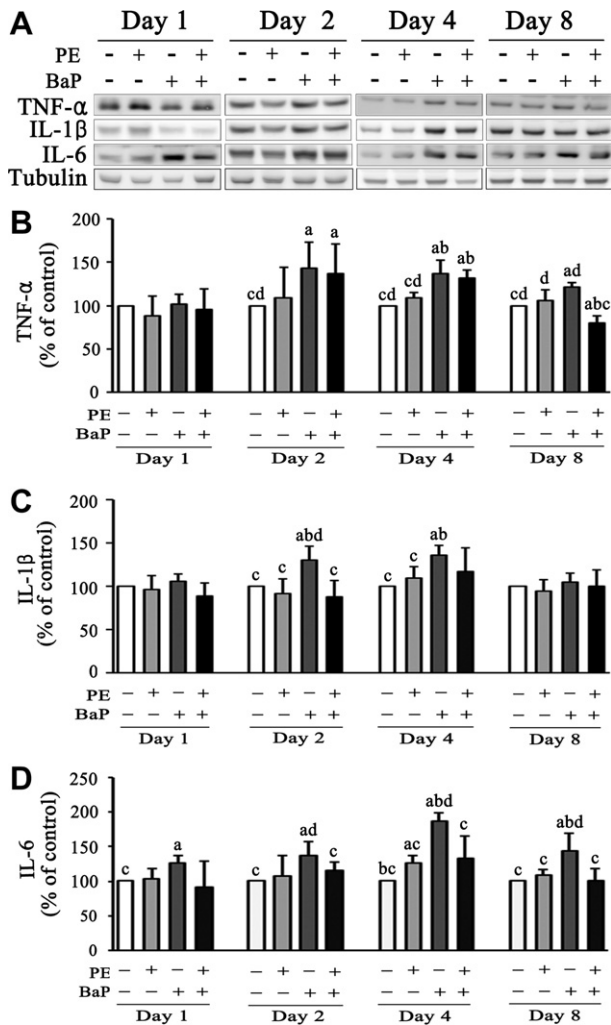
Treatment of rats with PE-only did not have a significant effect on the levels of IL-1 $\beta$  in all groups. Rats exposed to BaP for 2 or 4 days showed a significant increase in their levels of IL-1 $\beta$  compared to control rats, but rats exposed to BaP for 1 or 8 days showed no significant changes in the levels of IL-1 $\beta$ . The increased levels of IL-1 $\beta$  in rats exposed to BaP for 2 days were significantly attenuated by pre-treatment with PE, but the levels exposed to BaP for 4 days were not attenuated by pre-treatment with PE.

PE treatment itself did not have any effect on the expression levels of IL-6 in the all groups. BaP treatment of rats from all groups resulted in a significant increase in the levels of IL-6 in plasma. The increased level of IL-6 in rats exposed to BaP for 1 day was attenuated by pre-treatment with PE prior to BaP, but the effect was not statistically significant. However, the levels of IL-6 in rats exposed to BaP for 2, 4 or 8 days were significantly decreased by pre-treatment with PE.

## 4. Discussion

Human placenta has been used for years as a folk remedy in Asian countries. PE, which is the hydrolysate of human placenta, was approved for treatment of liver cirrhosis in Japan due to its





**Fig. 4.** The effect of placental extracts (PE) on pro-inflammatory cytokine levels in rats exposed to benzo[a]pyrene (BaP). (A) Western blot analysis of pro-inflammatory cytokines in plasma from rats exposed to BaP for 1, 2, 4, or 8 days with or without PE using anti-TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 antibodies. An equal amount of total protein was loaded into each lane. (B–D) Graphs showing changes in the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in plasma from rats exposed to BaP with or without PE. Densitometric values were normalized using tubulin as an internal control. Values are expressed as a percentage of control rats. All data represent the mean  $\pm$  SD of five different rats. <sup>a</sup> $p < 0.05$  compared to the control rats, <sup>b</sup> $p < 0.05$  compared to PE-treated rats, <sup>c</sup> $p < 0.05$  compared to BaP-treated rats, <sup>d</sup> $p < 0.05$  compared to PE + BaP-treated rats.

positive effects on liver regeneration [14,15]. Currently, PE is widely used in Korea for alleviation of fatigue, skin whitening, liver function, and menopausal symptoms [16]. Recent studies have reported the isolation of immunomodulatory peptide from bovine placenta water-soluble extract [17]. The isolation of a peptide having homology with human fibronectin type III was also reported as a principle for the wound healing properties of human placenta [18]. In addition, the anti-oxidative effects of PE can be attributed to amino acids such as tyrosine, phenylalanine and tryptophan [3]. Our sample analysis also indicated the presence of diverse amino acids, monosaccharides, and fatty acids in PE, suggesting that it had possible anti-oxidant, immunomodulating effects. For example, glutamic acids [19] and derivatives of aspartic acid and glutamic acid [20] identified in PE exhibited anti-oxidant activities that occurred via reductions in lipid peroxides or acting as free radical scavengers. In addition, N-acetylneuraminic acid [21], glucosamine [22], and glucose [23], which were identified as components of PE,

were also reported to exhibit anti-oxidant or anti-inflammatory properties in various biological systems. Furthermore, nucleotides have been reported to have anti-oxidant effects in Fe/Cu–H<sub>2</sub>O<sub>2</sub> systems that occur via acting as metal-ion chelators [24]. Diverse fatty acids, omega-3 fatty acids in particular, were also reported to have strong anti-oxidant and anti-inflammatory properties. For example, a recent clinical trial reported that omega-3 fatty acids significantly reduced oxidative stress in neonates when compared to standard lipids [25]. Moreover, omega-3 fatty acids decreased the expression of pro-inflammatory markers and increased the expression of anti-inflammatory markers in mice with *Pseudomonas aeruginosa* lung infection [26]. Taken together, these reports indicate that the anti-oxidant and anti-inflammatory effects of PE against BaP observed in the present study might be exerted by components of PE, including amino acids, monosaccharides and fatty acids. The major strength of this study is that it identifies the effect of PE on oxidative stress induced by the environmental contaminant, BaP. Most studies of PE that have been conducted to date have primarily focused on its anti-inflammatory effects. However, we demonstrated that the anti-oxidant effects of PE occur via recovery of DNA damage. In addition, the effects of PE on anti-oxidant enzymes such as SOD and lipid peroxidation confirmed the anti-oxidant effect of PE. Even though additional studies must be conducted to identify the detailed mechanisms by which PE recovers DNA damage, the anti-oxidant effect of PE could be an important basic concept to evaluate its biological activities.

BaP is one of the most carcinogenic PAHs. The metabolic activation of BaP by cytochrome P450 isoenzymes produces a variety of mutagenic or carcinogenic electrophiles. Particularly, benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), which is a metabolite of BaP, binds covalently to DNA, RNA, and proteins [27]. BPDE-DNA adducts have been shown to initiate proto-oncogenic *ras* [28] and may induce various cancers such as breast cancers [29]. BaP-quinones are also one of the BaP-metabolites produced by cytochrome P450 isoenzymes [30]. BaP-quinones are highly active and easily undergo one electron redox cycling resulting in the formation of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radicals [31] that have been associated with cell damage and apoptosis [32]. The DNA damage and oxidative stress induced by BaP in this study could therefore have been induced by metabolites of BaP such as BPDE or BaP-quinones rather than being a direct effect of BaP.

BaP-induced DNA damage has been reported in many different experimental systems including human hepatoma cell line HepG2 [33], Zebra mussels (*Dreissena polymorpha*) [34], and the human prostate carcinoma cell line DU145 [35]. It has been also reported that exposure of rats to BaP can induce a high level of DNA damage in peripheral lymphocytes based on Comet assays [36]. These results are in agreement with ours, suggesting that BaP induces DNA damage in rats.

PAHs including BaP have been proposed to be immunotoxic based on human monitoring. Coke oven workers in Poland who are chronically exposed to PAHs show marked depression of serum IgG and IgA levels compared to other workers [37]. However, Winker et al. reported that serum IgG levels of the PAH-exposed group are not significantly lower than those of control [38]. In our study, exposure of rats to BaP for 1, 2, 4 or 8 days did not suppress the expression of immunoglobulins such as IgG1 and IgG2a.

Pro-inflammatory cytokines such as IL-1 $\beta$  and IFN- $\gamma$  have been implicated in the inflammatory effects in BaP-exposed cells [39]. Increased levels of IL-6 have also been reported in rats exposed to BaP [40]. However, conflicting results regarding cytokine TNF- $\alpha$  have been reported. Treatment of primary human macrophages with BaP increased the level of TNF- $\alpha$  significantly [41]. In contrast, TNF- $\alpha$  secretion was unchanged in the murine macrophage cell line

RAW 264.7 treated with BaP [42]. These contradictory results may be due to species or cell type-specific differences. In this study, exposure of rats to BaP resulted in a significant increase in the levels of pro-inflammatory cytokines.

One of the most important roles of the placenta is to protect the embryo(s) from oxidative stress [43]. Therefore, PE is known to have anti-oxidative properties [44]. The major anti-oxidant components in PE are uracil, tyrosine, phenylalanine, and tryptophan. About 59% of the anti-oxidative effects of PE can be explained by those components [3]. Togashi et al. reported that peptides produced from collagen are also anti-oxidative components of PE [4]. In this study, PE significantly decreased BaP-induced oxidative stress which was evaluated by measuring the levels of SOD and lipid peroxidation [4].

Exposure of PE significantly attenuated carrageenin and prostaglandin E1-induced edema in rats and this effect was almost the same as in the anti-inflammatory drug-treated groups, suggesting the anti-inflammatory properties [45]. In this study, PE pre-treatment significantly decreased the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in rats exposed to BaP. These results suggest that PE attenuates the levels of pro-inflammatory cytokines and decreases the inflammation induced by BaP. To the best of our knowledge, this is the first report to show that PE decreases the expression of pro-inflammatory cytokines induced by BaP.

In this study, we evaluated the protective effects of PE on rats exposed to BaP. Exposure of rats to BaP significantly increased the levels of Olive Tailmoments above control levels, while pre-treatment with PE prior to BaP exposure significantly decreased the Olive Tailmoments, suggesting that PE protects against DNA damage induced by BaP. In addition, oxidative stress induced by BaP was attenuated by pre-treatment with PE, as examined by measuring the levels of SOD and lipid peroxidation. Furthermore, PE pre-treatment significantly decreased the levels of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Taken together, these results suggest that pre-treatment of rats with PE can significantly attenuate the oxidative damage and inflammation induced by BaP. However, the protective effects of PE against environmental toxicants in humans need to be elucidated further.

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