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SHORT COMMUNICATION

Anti-inflammatory and analgesic effects of human placenta extract

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In this study, we investigated the effects of human placenta extract (HPE, Laennec inj.) on pro-inflammatory cytokines and mediators secreted from lipopolysaccharide-stimulated RAW264.7 macrophages. We found that HPE significantly inhibited the production of nitric oxide, tumour necrosis factor-α and cyclooxygenase-2. We studied the anti-inflammatory and analgesic potential of HPE in murine models of inflammation/inflammatory pain. Rats were assigned to six groups and were administered either saline or HPE (0.33, 1, 3 and 6 mL kg^{-1}) intraperitoneally. Diclofenac was used as a positive control. HPE attenuated the swelling of the rat's hind paw. The vascular permeability induced by acetic acid was significantly reduced by HPE. HPE reduced the formation of granuloma in carrageenan air pouch and hind paw oedema in complete Freund's adjuvant-induced chronic arthritis in rats. HPE attenuated writhing episodes. An increase in hot-plate latency was observed in mice receiving HPE. HPE also increased the pain threshold in the Randall–Selitto test. In the tail-flick assay, HPE prolonged the reaction time of rats to radiant heat stimulation. These results suggest that HPE has potent anti-inflammatory and anti-nociceptive activities.

Keywords: acute inflammation; analgesic; chronic inflammation; granuloma; human placenta extract; pro-inflammatory cytokines

1. Introduction

Inflammation is a beneficial host response to a variety of stimuli and local injury (Sobota, Szwed, Kasza, Bugno, & Kordula, 2000). The inflammatory reaction can be triggered by physical or chemical trauma, invading organisms and antigen–antibody reactions, and is often exacerbated by the resultant swelling or oedema of tissues, pain or even cell damage (Yam, Asmawi, & Basir, 2008). Hence, the use of anti-inflammatory agents may be helpful in the therapeutic treatment of pathologies associated with the inflammatory reaction. However, the side effects of the currently available anti-inflammatory drugs pose major complications in their clinical use. For instance, some non-steroidal anti-inflammatory drugs may cause gastric ulceration and renal damage (Perry, 1998). Therefore, anti-inflammatory drugs with less severe

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side effects must be developed. There is recent interest in natural products, and many natural products are attractive as source materials for the development of new medicines.

Human placenta extract (HPE, Laennec inj.) has been used as a traditional therapeutic agent in many Asian countries for treatment of diseases or replenishment of vital essence (Yeom et al., 2003). The therapeutic effects of HPE on chronic liver diseases and inflammatory skin diseases such as psoriasis have also been reported (Banerjee, Bishayee, & Chatterjee, 1995). Recently, HPE was shown to improve menopausal symptoms and fatigue in middle-aged women (Kong et al., 2008) and to alleviate the arthritic symptoms in adjuvant-induced arthritic rats (AIA; Yeom et al., 2003). In addition, HPE has been shown to possess anti-oxidative activities that counteract the adverse consequences of oxidative stress, which interferes with normal metabolism and physiological balance by damaging biological molecules including proteins, lipids and DNA (Halliwell, 1997). HPE promotes Interleukin-8 (IL-8) expression through activation of Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells and activating protein-1 (NF-κB and AP-1) in a human monocytic cell line (Kang et al., 2007). Although HPE has been widely applied in various clinical settings, limited information is available on the precise mechanisms of its anti-inflammatory properties and related therapeutic efficacy.

Therefore, in this study, we examined the anti-inflammatory and anti-nociceptive properties of HPE in several experimental models.

2. Results

2.1. *Cell viability*

The cytotoxic effects of HPE are shown in Supplementary Figure S1 (online only). No cytotoxic effect was observed upto a concentration of 3.6 mL^{-1}.

2.2. *Effects of HPE on LPS-induced nitrite and cytokine release in RAW 264.7 cell*

NO, produced by macrophages, increased from 0.64 to 34.71 μmol after LPS exposure. This change was significantly inhibited by HPE of 1.8 and 3.6 mL^{-1} (Supplementary Figure S2a – online only). We also investigated the effect of HPE on the production of TNF-α, IL-1β and IL-6. As shown in Supplementary Figure S2(b), the levels of TNF-α, IL-1β and IL-6 were significantly increased by LPS. HPE dose-dependently inhibited the increase in the level of TNF-α. HPE also inhibited the increases in the level of IL-1β and IL-6 at the concentration of 3.6 mL^{-1}.

2.3. *Effects of HPE on LPS-induced protein expression of iNOS and COX-2 in RAW 264.7 cell*

The levels of iNOS and COX-2 protein expression in LPS-treated group were approximately five- and four-folds higher than the control level, respectively. The increases in iNOS and COX-2 protein expression were significantly suppressed by HPE in a concentration-dependent manner (Supplementary Figure S3 – online only).

2.4. *Effects of HPE on carrageenan-induced oedema in the hind paw*

Subplantar injection of carrageenan in rats resulted in a time-dependent increase in paw volume in the vehicle-treated control group (Supplementary Figure S4 – online only); this increase was observed at 1 h and was maximal at 6 h after injection of carrageenan injection. HPE at a dose of 0.33 mL kg^{-1} did not affect the paw volume compared with the vehicle-treated control group. However, at doses of 1, 3 and 6 mL kg^{-1}, HPE significantly reduced this increase. Diclofenac, used as a positive control, also showed a significant anti-inflammatory effect.

2.5. *Effects of HPE on acetic acid-induced vascular permeability*

As shown in Supplementary Figure S5 (online only), dye leakage induced by acetic acid was 31.6 ± 2.6 μg 10 g^{-1} body weight in the vehicle-treated control group. No apparent changes in dye leakage were observed at HPE doses of 0.33, 1 and 3 mL kg^{-1}. However, 6 mL kg^{-1} of HPE significantly reduced the dye leakage into the peritoneal cavity.

2.6. *Effects of HPE on carrageenan-induced air pouch*

In the vehicle-treated control animals, the volume of exudates was 6.3 ± 0.8 mL and the granuloma formation was 2.0 ± 0.2 g. There were no significant changes in the volume of exudates among any of the experimental groups. The granuloma formation was significantly attenuated by 1, 3 and 6 mL kg^{-1} of HPE (Supplementary Table S1 – online only).

2.7. *Effects of HPE on AIA*

In the vehicle-treated control group, the peak paw volume was observed on day 8; paw volume then decreased until day 20. Until day 5 after HPE treatment, there were no considerable changes compared with the vehicle-treated control group and paw volume markedly decreased from day 18 to day 20 at doses of 0.33, 1 and 3 mL kg^{-1} of HPE. At a dose of 6 mL kg^{-1}, HPE significantly decreased the paw volume at day 20 (Supplementary Figure S6 – online only).

2.8. *Effects of HPE on acetic acid-induced writhing response*

The number of writhings was 18.0 ± 1.6 in the vehicle-treated control group. Treatment of animals with HPE at doses of 0.33, 1 and 3 mL kg^{-1} significantly decreased abdominal writhing produced by acetic acid. Aminopyrine used as a positive control also significantly reduced the writhing counts (Supplementary Table S2 – online only).

2.9. *Effects of HPE on Randall–Selitto assay*

The pain threshold of inflamed rat paw was steady at all test times in the vehicle-treated control group. HPE at doses of 0.33 and 1 mL kg^{-1} did not increase the pain

threshold. However, at doses of 3 and 6 mL kg^{-1}, HPE caused a significant increase in nociceptive threshold compared with the vehicle-treated control group (Supplementary Figure S7 – online only).

2.10. *Effects of HPE on tail-flick test*

In the vehicle-treated group, the reaction time remained low throughout the experiment. At a dose of 0.33 mL kg^{-1}, HPE significantly increased the reaction time at 5, 30 and 60 min; at a dose of 1 mL kg^{-1}, HPE significantly increased the reaction time only at 5 min. At doses of 3 mL kg^{-1} and 6 mL kg^{-1}, HPE significantly increased the nociceptive threshold (Supplementary Figure S8 – online only).

2.11. *Effects of HPE on hot-plate test*

The response time was 13.8 ± 1.6 s in the vehicle-treated control group (Supplementary Table S3 – online only). At doses of 0.33, 1 and 3 mL kg^{-1}, HPE exhibited anti-nociceptive effects by increasing the latency time to 25.2, 23.6 and 25.0 s, respectively, which differed significantly from the vehicle-treated control group.

3. Discussion

In this study, the anti-inflammatory and anti-nociceptive activities of HPE were investigated using *in vitro* cell culture and *in vivo* animal models.

LPS-stimulated RAW264.7 mouse macrophage cell has been used as an inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs (Kim et al., 2003). Activation of macrophages plays an important role in the initiation and propagation of inflammatory responses by production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, as well as inflammatory mediators, including NO, which is synthesised by iNOS, and COX-2 (Chan & Riches, 2001). So these key inflammation cytokines and mediators were evaluated in LPS-stimulated RAW264.7 cells to make the anti-inflammatory effects of HPE clear. HPE showed no cytotoxicity against murine macrophage, but displayed inhibitory effects on LPS-induced NO, TNF-α and COX-2 production. Thus, our results suggest that HPE has anti-inflammatory activity related with its inhibition of NO, TNF-α and COX-2 production.

The carrageenan-induced rat paw oedema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the anti-oedematous effects. Oedema development in the paw of the rat after carrageenan injection is a biphasic event (Vinegar, Schreiber, & Hugo, 1969). The initial phase observed during the first hour is attributed to the release of histamine and serotonin and the second phase of oedema is due to the release of prostaglandin (Di Rosa, Giroud, & Willoughby, 1971). In the vehicle-treated control group, the paw volume of rat significantly increased after injection of carrageenan, indicating acute inflammatory damage. In contrast, treatment with HPE markedly attenuated the swelling of rat hind paw. These results suggest that HPE may have potential clinical applications for treating inflammatory disorders.

Increased vascular permeability is one of the essential features of the acute inflammatory response. Mediators of inflammation such as histamine, prostaglandins and leukotrienes are released following stimulation (Hseu et al., 2005). This leads to dilation of arterioles and venules and to increased vascular permeability (H. Vogel & W. Vogel, 1997); as a result, fluid and plasma protein are extravasated to the abdominal cavity and oedemas form. HPE reduced the intensity of the peritoneal inflammation produced by acetic acid, indicating that it may have the ability to inhibit the permeability of small blood vessels during acute inflammation.

In vivo anti-inflammatory effects of HPE were also tested in animal models of subchronic inflammation. This model was characterised by exudate volume, leukocyte accumulation, granuloma, vascular permeability and protein clearance up to 7 days after injection of carrageenan (Hambleton & Miller, 1989). HPE significantly inhibited granuloma formation. This result reflects the ability of HPE to inhibit the proliferation phase of the inflammation process.

We investigated whether the HPE was able to exert anti-inflammatory effects in a chronic inflammatory disease model, i.e. the rat AIA model. HPE significantly inhibited the development of chronic joint swelling induced by the adjuvant. Injection of complete Freund's adjuvant into the rat's hind paw induces inflammation at the primary lesion; this inflammation peaks after 3–5 days. Secondary lesions occur after injection, i.e. a decrease in weight and reduced immune response. It appears that bacterial peptidoglycan and muramyl dipeptide are responsible for the induction of these symptoms (Crofford, 1993). In tests of short duration (such as those involving carrageenan-induced oedema in rats) as well as in tests of longer duration (such as those based on complete Freund's AIA in rats), HPE exhibited significant anti-inflammatory effects. These results indicate that HPE is effective for the treatment of not only acute but also chronic inflammation.

HPE exhibited analgesic activity when assessed in abdominal constriction, hot-plate, Randall–Selitto and tail-flick tests. Acetic acid causes algesia due to the liberation of endogenous substances (Berkenkopf & Weichman, 1988). This method has been associated with prostanoids in general, e.g. increased levels of PGE_2 and $PGF_{2\alpha}$, in peritoneal fluids (Deraedt, Jouquey, Delevallee, & Flahaut, 1980) as well as lipoxygenase products (Dhara, Suba, Sen, Pal, & Chaudhuri, 2000). In the paw pressure test developed by Randall and Selitto, half of the oedematous response to yeast results from direct capillary changes produced by released 5-HT (Stone, Wenger, Ludden, Starvorski, & Ross, 1961). Inflammation and strong hyperalgesia develop 1–3 h after yeast is injected (Winter & Flataker, 1965). HPE exerted significant inhibitory effects on the writhing response and increased the pain threshold, signifying peripherally mediated activities.

To assess the central mechanism of HPE in producing acute analgesia, the hot-plate and tail-flick methods were employed. In these methods, pain is mediated through a central mechanism. These methods can be further distinguished by the response to pain stimuli conducted through different pathways. The tail-flick response is mediated by a spinal reflex to nociceptive action, while the hot-plate response involves higher brain centres and is considered a supraspinally organised response (Chapman et al., 1985). HPE significantly increased the reaction time in the hot-plate test and the withdrawal latency time in the tail-flick test, thereby indicating its central action in producing an analgesic effect.

Overall, HPE appears to have potent anti-inflammatory and analgesic activities, indicating that HPE could have therapeutic applications in various diseases associated with inflammation.

4. Experimental

4.1. *Chemicals*

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (PBS), penicillin/streptomycin (1000 U mL^{-1}, 1000 µg mL^{-1}, respectively) and foetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (Grand Island, NY, USA). Lipopolysaccharide (LPS; *Escherichia coli* serotype 0127:B8), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), and all the other materials required for culturing of cells were purchased from Sigma Chemical Company (St Louis, MO, USA). Type IV lambda carrageenan, Brewer's yeast, complete Freund's adjuvant, diclofenac and Evans blue were supplied by Sigma Chemical Co. (USA). All the other chemicals used in this study were of reagent grade and were locally and commercially available.

4.2. *Preparation and composition of HPE*

We used Laennec, the hydrolysate of human placenta commercially marketed under the trade name HPE, supplied by Green Cross Japan Bio Products Ltd. (Korea). Human placentas collected upon full-term delivery were immediately placed in ice. They were tested for human immunodeficiency virus and hepatitis B and C viruses and then cut into pieces and extracted with water using enzymatic molecular separation and chemical hydrolysis. Extraction and sterilisation were performed under the quality control guidelines. The final products of HPE were stored in 2 mL ampules. Each ampule contained various amino acids including arginine (0.08%), lysine (0.1%), phenylalanine (0.08%), tyrosine (0.03%), leucine (0.12%), methionine (0.03%), valine (0.04%), alanine (0.08%), serine (0.07%) and threonine (0.06%). Insoluble macromolecules such as polysaccharides and polynucleotides were excluded during the manufacturing process.

4.3. *Cell culture*

Macrophage RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). These cells were maintained at subconfluence in 95% air, 5% CO_2 humidified atmosphere at 37°C. The medium for routine subculture was DMEM supplemented with 10% FBS, 2 mmol glutamine and 100 µg mL^{-1} penicillin/streptomycin.

4.4. *Cell viability*

The effect of the concentration of HPE on cell viability was assessed using a modified MTT assay (Mosmann, 1983). Briefly, cells (1×10^4 cells per well) were seeded in a 96-well plate and incubated at 37°C for 24 h. The cells were treated with various

concentrations of HPE or a vehicle (PBS) and positive controls alone and were incubated at 37°C for additional 24 h. After incubation, 100 μL of MTT solution (5 mg mL^{-1} in PBS) was added to each well and the plates was further incubated for 3 h at 37°C. Subsequently, 100 μL of dimethyl sulphoxide was added to each well to solubilise any deposited formazon. The optical density of each well was measured at 450 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA).

4.5. *Nitrite assay*

Nitrite concentration in the medium was measured as an indicator of nitric oxide (NO) production according to the Griess method reported by Hishikawa, Nakaki, Suzuki, Saruta, and Kato (1992). In brief, RAW264.7 cells were plated at a density of 1×10^5 cells mL^{-1} in a 24-well cell culture plate with 500 μL of culture medium, incubated for 24 h, pre-treated with indomethacin (50 μmol), dexamethasone (1 μmol) or HPE (0.0018, 0.018, 0.18 and 3.6 mL^{-1}) for another 2 h, and then challenged with LPS (1 μg mL^{-1}) for an additional 24 h. Equal volumes of culture medium and Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed, and the absorbance of the mixture at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA).

4.6. *Measurement of cytokines by enzyme-linked immunoassay*

RAW264.7 cells were plated at a density of 1×10^6 cells mL^{-1} in a six-well cell culture plate with 2 mL of culture medium, incubated for 24 h, pre-treated with indomethacin (50 μmol), dexamethasone (1 μmol) or HPE (0.0018, 0.018, 0.18 and 3.6 mL^{-1}) for another 2 h, and then challenged with LPS (1 μg mL^{-1}) for an additional 24 h. Inflammatory mediators such as tumour necrosis factor-α (TNF-α), IL-1β and IL-6 were quantified at 24 h after LPS treatment by commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

4.7. *Western blot assay*

RAW264.7 cells were pre-treated with indomethacin (50 μmol), dexamethasone (1 μmol) or HPE (0.0018, 0.018, 0.18 and 3.6 mL^{-1}) for 2 h, and stimulated with LPS (1 μg mL^{-1}) for 24 h. Twenty micrograms of total protein extracts was separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and electro-transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were blocked with 5% non-fat milk for 1 h and then incubated overnight with polyclonal antibody for inducible nitric oxide synthase (iNOS, Transduction Laboratories, San Jose, CA, USA) and cyclooxygenase-2 (COX-2, Cayman, Ann Arbor, MI, USA) and monoclonal antibody for β-actin (Sigma, St. Louis, MO, USA). The binding of all the antibodies was detected using an enhanced chemiluminescence (ECL) detection system (iNtRON Biotechnology, Seoul, Korea), according to the manufacturer's instructions. The intensity of the

immunoreactive bands was determined using a densitometric analysis program (Image Gauge V3.12, FUJIFILM, Japan).

4.8. *Treatment of animals*

Male Sprague-Dawley rats (150–250 g) and imprinting control region (ICR) mice (25–28 g) were obtained from DaeHan Biolink Co. (Korea) and were acclimated to the laboratory conditions for at least 1 week. All animals were treated humanely under the Sungkyunkwan University Animal Care Committee guidelines. HPE was diluted in saline (vehicle) and administered at doses of 0.33, 1, 3 and 6 mL kg^{-1}. Six treatment groups ($n = 8$–16) were examined: (1) vehicle-treated control; (2–5) HPE-treated groups; (6) positive drug (diclofenac)-treated group. This HPE dosing regimen was selected because it had been evaluated previously (Liu, Yoshii, & Kawabata, 2004).

4.9. *Carrageenan-induced hind paw oedema*

The carrageenan-induced paw oedema described by Winter, Risley, and Nuss (1962) was used with slight modifications. HPE and were administered by intraperitoneal (i.p.) injection 30 min before subplantar injection of 0.1 mL of 1% sodium carrageenan into the rat's right hind paw. Right paw volumes were measured immediately and at 0.5, 1, 2, 4, 6, 8, 10 and 12 h after injection of carrageenan using a water plethysmometer from Ugo Basile (Italy). The rate of paw oedema was calculated according to the following equation:

$$\text{Oedema rate } (\%) = (B - A)/A \times 100\%,$$

where B is the volume of the paw after carrageenan injection and A is that before the injection.

4.10. *Acetic acid-induced vascular permeability*

After 30 min of intramuscular injection of HPE, 2.5% Evans blue solution was intravenously injected into mice and 20 min later, 10 μL g^{-1} body weight of 0.6% of acetic acid solution was intraperitoneally injected. After another 20 min, mice were sacrificed and the peritoneal cavity was washed with saline. The absorbance of the supernatant of the peritoneal cavity fluid was measured at 630 nm using a spectrophotometer (Whittle, 1964).

4.11. *Carrageenan-induced air pouch*

8 mL of air was injected into the dorsal subcutaneous space of rats to create a pouch. 0.2% carrageenan-saline solution was injected into the pouch 24 h after the air injection (Hambleton & Miller, 1989). Vehicle, HPE or diclofenac (5 mg kg^{-1}) were intraperitoneally administered once-daily at the same time for 7 days. After 24 h of final treatment, animals were sacrificed and the exudates and granuloma were collected and measured.

4.12. *Adjuvant-induced arthritis*

According to the method reported by Cox and Welch (2004), animals were subcutaneously injected in the base of the right hind paw with 1 mg of heat-killed *Mycobacterium butyricum* in 1 mL of complete Freund's adjuvant. After 11 days of adjuvant injection, HPE was intraperitoneally administered daily for 10 days. Arthritis was assessed by measuring the degree of swelling in the paw with a plethysmometer. The oedema rate was measured as described above.

4.13. *Acetic acid-induced writhing response*

HPE was intramuscularly administered 30 min before the experiment, and acetic acid in saline (0.7%, 0.1 mL 10 g^{-1} body weight) was then injected intraperitoneally. After 5 min of the IP injection of acetic acid, the number of writhings was counted for the next 10 min (Koster, Anderson, & de Beer, 1959).

4.14. *Randall–Selitto assay*

A 20% suspension of Brewer's yeast (0.1 mL) was injected into the plantar of the hind paw of rats. Two hours after the injection of Brewer's yeast, HPE was intraperitoneally administered. Subsequently, the nociceptive threshold of both the inflamed and intact paws of each group was measured using an analgesymeter at 0.5, 1, 2, 3 and 4 h after treatment. The nociceptive threshold was defined as the force at which the rat vocalised or withdrew its paw (Randall & Selitto, 1957).

4.15. *Tail-flick test*

Tail-flick latencies were determined 5, 15, 30, 60, 90, 120 and 180 min following i.p. injection of HPE. The heat was focussed approximately midway along the tail and the latency time for removal of the tail from the stimulus was recorded. A 15 s cutoff was imposed to prevent tissue damage (Minami et al., 2009).

4.16. *Hot-plate test*

For the hot-plate test, the temperature of the hot-plate was set at 55°C ± 0.5°C. Thirty minutes after i.p. injection of HPE, the mice were placed on the hot-plate and the latency time that elapsed until the animal jumped or licked one of its hind paws was recorded in seconds. A cut-off time of 30 s was imposed to prevent tissue damage (Woolfe & MacDonald, 1944).

4.17. *Statistical analysis*

Overall significance was tested by one-way analysis of variance (ANOVA). Differences between groups at specific time points were considered significant at $p < 0.05$, with the appropriate Bonferroni correction made for multiple comparisons. All results are presented as means ± standard error mean (SEM).

Supplementary material

Tables S1–S3 and Figures S1–S8 relating to this paper are available online.

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