

Original Article

Anti-inflammatory effects of porcine placenta in forced swimming tested fatigue mice and RAW264.7 cells

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ABSTRACT

Inflammation has been linked to various diseases. Especially, fatigue is a frequent symptom in several inflammatory disorders. Therefore, blocking inflammatory process is effective in fatigue. We investigated whether Denmark porcine placenta (DPP) alleviates fatigue by inhibiting inflammatory reaction using forced swimming test (FST) animal model and RAW264.7 cells. In FST-induced fatigue animal model, the mice which received the DPP for 21 days showed decreases of interleukin (IL)-1 β and IL-6 serum levels. Furthermore, our data revealed that lipopolysaccharide (LPS)-induced IL-1 β , IL-6, and tumor necrosis factor- α secretion were markedly inhibited by DPP in RAW264.7 cells without inducing cytotoxicity. LPS-enhanced nitric oxide secretion and inducible nitric oxide synthase expression were inhibited by DPP. The present study also figured out that these effects of DPP were mediated by blockade of caspase-1 and nuclear factor- κ B activation. Taken together, our results indicated that DPP could be alleviating fatigue as candidate of anti-inflammatory agent.

Keywords Denmark porcine placenta, fatigue, RAW264.7 cells, inflammatory cytokines, nitric oxide, caspase-1, NF- κ B

INTRODUCTION

Inflammation is an important biological defense response against various stimuli, such as damaged cells, pathogens, or irritants. Several inflammatory illnesses like inflammatory bowel disease, rheumatoid arthritis, liver disease, psoriasis, and cancer are closely linked to fatigue (Norheim et al., 2011). Fatigue is one of the most common debilitating symptoms with a significant economic global burden to society. It affects approximately 0.2 - 2.6% worldwide in adult population (Nacul et al., 2011; Prins et al., 2006). Fatigue is characterized by impaired activities of daily living and occupational ability as well as physical and social functioning (Ouellet et al., 2006; Stulemeijer et al., 2006). Terms of the exact pathophysiological mechanisms contributing to the fatigue are not clearly delineated, but multiple lines of evidence points to pro-inflammatory cytokines as important mediators (Norheim et al., 2012; Harboe et al., 2009). Certain proinflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF)- α , and IL-6 was involved in fatigue (Schubert et al., 2007). Exogenous administration of pro-inflammatory cytokines, including interferon gamma (IFN- γ), TNF- α , IL-1, IL-6, and IL-2 accelerated fatigue in healthy individuals (Bluthé et al., 1994; Dantzer et al., 2008; Kerr et al., 2001). Kerr et al. (2001) studied that parvovirus B19 induced raised levels of TNF- α in individual were closely linked to the feelings of fatigue. In addition, administration of IFN, IL-6, IL-12, TNF- α , IL-1 β , and

IL-2 to human, and IL-1 to animals clearly showed the development of cancer-related fatigue along with other illness symptoms (Kelly et al., 2003). The fact that these symptoms can be antagonized by the administration of IL-1RA has been demonstrated by earlier biological research (Swiergiel et al., 1997).

The placenta is a storehouse of potent active molecules, such as cytokines, hormones, growth factors, and chemokines (Pal et al., 2002; Tonello et al., 1996). Traditionally, placenta extract has been available clinically as a functional food or medicine in Japan, China, and Korea to treat various immune disorders (Pal et al., 2002; Yeom et al., 2003). Therapeutic effects of placenta extract on psoriasis, wound healing, and rheumatoid arthritis have also been studied (Kang et al., 2007; Tonello et al., 1996; Yeom et al., 2003). In addition, a small pilot study showed that placenta has been shown to have antioxidant abilities (Togashi et al., 2002). Recently, we reported an anti-fatigue effect of fermented porcine placenta. The studies presented here further describe the anti-inflammatory efficacy and underlying mechanisms of porcine placenta in force swimming test (FST) animal model and LPS-stimulated RAW264.7 cells.

MATERIAL AND METHODS

Reagents

LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and sodium nitrite were purchased from Sigma Chemical Co. (St Louis, MO, USA). Murine recombinant TNF- α , IL-1 β , and IL-6, anti-mouse TNF- α , IL-1 β , and IL-6, and biotinylated mouse TNF- α , IL-1 β , and IL-6, and were purchased from R&D Systems (Minneapolis, Minnesota, USA). Antibodies of caspase-1,

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Table 1. DPP relieved the levels of proinflammatory cytokines in the serum of FST-induced fatigue mice

	IL-1 β	IL-6	TNF- α
DW	2.7 $\pm$ 0.14	80.83 $\pm$ 5.71	1.86 $\pm$ 0.06
DPP (1 mg/kg)	2.0 $\pm$ 0.22*	11.35 $\pm$ 2.40*	1.71 $\pm$ 0.21
DPP (10 mg/kg)	2.05 $\pm$ 0.26*	8.54 $\pm$ 3.58*	1.89 $\pm$ 0.20

After the first measurement of immobility times, the mice were divided into DW (control) and DPP (1 and 10 mg/kg) based on the recorded swimming times (n = 5/group). DW or DPP (1 and 10 mg/kg) groups were orally administered to mice once per day for 21 days. After the FST, the levels of IL-1 β , IL-6, and TNF- α in the serum were analyzed by ELISA method. Values are the mean $\pm$ SEM. **p* < 0.05, significantly different from the control mice. DW, distilled water; DPP, Denmark porcine placenta.

inducible nitric oxide synthase (iNOS), nuclear factor- κ B (NF- κ B), phosphorylated (p)I κ B- α , poly (ADP-ribose) polymerase (PARP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, New York, USA). Denmark porcine placenta (DPP) was purchased from BIOFAC A/S (Denmark) and dissolved in distilled water (DW) and prepared at a dose of 10 μ g/ml according to previous report (Han et al., 2013).

Animals

Male ICR mice (3 weeks old, 10 - 12 g) were purchased from the Dae-Han Experimental Animal Center (Daejeon, Korea), and subsequently maintained at the College of Korean Medicine, Kyung Hee University. Experiments were initiated after 1 week to allow for adaption to the laboratory environment. The research was conducted in accordance with guidelines from the Canadian Council on Animal Care (CCAC).

FST

The mice were divided into DW (control) or DPP (1 and 10 mg/kg) groups and DW or DPP (1 and 10 mg/kg) groups were orally administered to mice once per day for 21 days. The FST was carried out once a week according to Han et al. (2013). After last FST, mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). Blood was withdrawn from the hearts of forced swimming-tested mice. Then, the serum was prepared by centrifugation at 3000 rpm at 4°C for 10 min.

Cell culture

RAW264.7 cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat inactivated FBS at 37°C, 5% CO₂, and 95% humidity. DPP were diluted with DMEM containing 10% FBS. Cells were pretreated with DPP (0.1, 1, and 10 μ g/ml) for 1 h prior to LPS stimulation.

Assay of cytokine release

Cytokines of serum and supernatant were measured by a modified enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's specifications (R&D systems and BD Pharmingen). MTT was performed according to a previous report (Jeong et al., 2014).

Measurement of nitrite concentration

NO assay was performed according to a previous report (Jeong

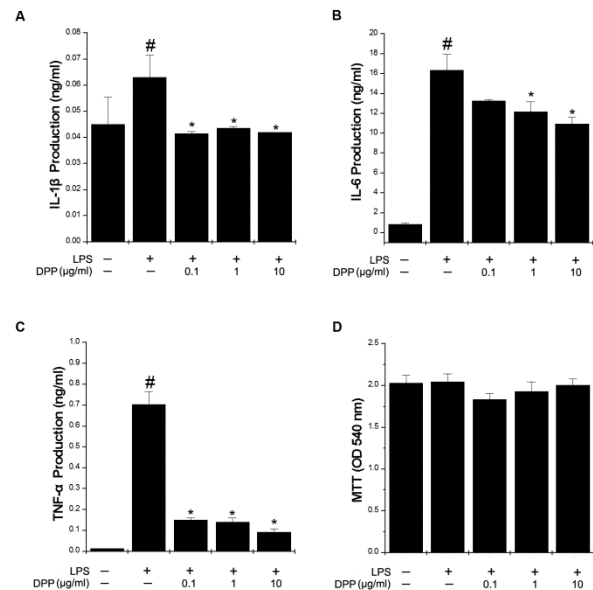


Fig. 1. DPP relieved the secretion of proinflammatory cytokines in the activated RAW264.7 cells. Cells were pretreated with DPP for 1 h and then stimulated with LPS (10 μ g/ml) for 24 h. (A-C) The levels of IL-1 β , IL-6, and TNF- α were measured by ELISA method. (D) MTT assay. Data are mean $\pm$ SEM values of three independent experiments performed in duplicate. #*p* < 0.05: significantly different from the unstimulated cells; **p* < 0.05: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; DPP, Denmark porcine placenta. MTT assay

et al., 2014).

Western blot analysis

Western blot analysis was performed as previously reported (Jeong et al., 2002). Briefly, proteins in the cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 6% bovine serum albumin in PBS and then incubated with antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with a 1:3000 dilution of horseradish peroxidase-linked whole antibodies (Amersham Corp. Newark, NJ, USA). Antibody-specific proteins were visualized using the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Caspase-1 assay

The enzymatic activity of caspase-1 was assayed using a colorimetric assay kit according to the manufacturer's protocol.

Statistical analysis

In vitro data are presented as means $\pm$ standard error of means (SEMs) of three independent experiments performed in duplicate. In vivo data are expressed as the mean $\pm$ SEM (n = 5). Statistical evaluation of the results was performed by an independent t-test and ANOVA with Tukey's post hoc test. All statistical analyses were performed using SPSS v12.00 statistical analysis software (SPSS Inc.). The results were considered significant at a value of *p* < 0.05.

RESULTS

DPP relieved the levels of proinflammatory cytokines in the serum of FST-induced fatigue mice

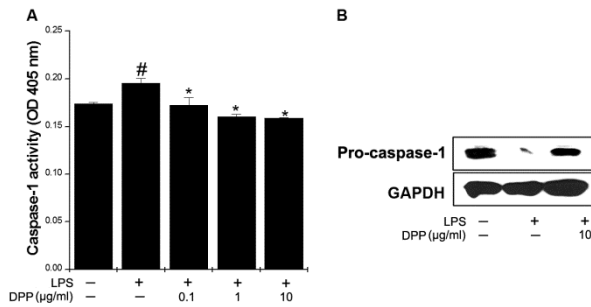


Fig. 2. DPP relieved the caspase-1 activity in the activated RAW264.7 cells. Cells were pretreated with DPP for 1 h and then stimulated with LPS (10 μg/ml) for 2 h. (A) Caspase-1 activity was measured by a caspase-1 assay kit. Data are mean ± SEM values of three independent experiments performed in duplicate. (B) Caspase-1 protein levels were analyzed by Western blotting. GAPDH was used as a loading control. [#] $p < 0.05$: significantly different from the unstimulated cells; ^{*} $p < 0.05$: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; DPP, Denmark porcine placenta.

To investigate the effect of DPP on the relationship between inflammatory cytokine levels and fatigue, FST-induced proinflammatory cytokines levels were detected firstly. As shown in Table 1, compared to the control group, the levels of IL-1β and IL-6 were significantly decreased in DPP-treated group ($p < 0.05$). However, DPP failed to decrease TNF-α levels (Table 1).

DPP relieved the secretion of proinflammatory cytokines in the activated RAW264.7 cells

Regarding proinflammatory cytokine secretion, we investigated the effect of DPP on secreted IL-1β, TNF-α, and IL-6 protein levels in the activated RAW264.7 cells. After incubating cells with various doses of DPP in the presence or absence of LPS, assessment of IL-1β levels showed that LPS-induced IL-1β secretion was markedly suppressed by treatment of DPP (Fig. 1A, $p < 0.05$). Similarly, significant enhancement of TNF-α and IL-6 secretion were observed by LPS treatment, but DPP demonstrated a significant decrease in TNF-α and IL-6 (Fig. 1B and C, $p < 0.05$). Next, we explored whether DPP has any cytotoxicity on RAW264.7 cells. The influence of DPP on cell

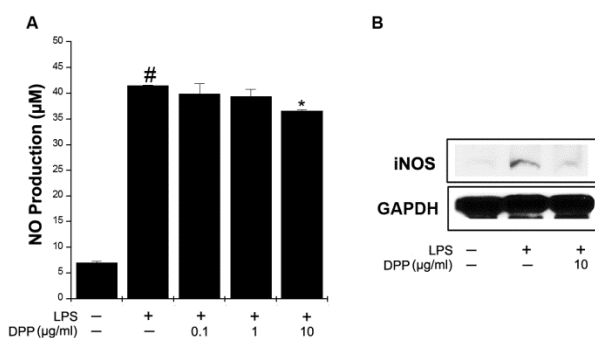


Fig. 3. DPP relieved the secretion of NO and expression of iNOS in the activated RAW264.7 cells. Cells were pretreated with DPP for 1 h and then stimulated with LPS (10 μg/ml) for 48 h. (A) NO production was measured by Griess method. (B) Cells were pretreated with DPP for 1 h and then stimulated with LPS (10 μg/ml) for 24 h. The total proteins were determined for iNOS expression by Western blotting. GAPDH was used as a loading control. Data are mean ± SEM values of three independent experiments performed in duplicate. [#] $p < 0.05$: significantly different from the unstimulated cells; ^{*} $p < 0.05$: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; DPP, Denmark porcine placenta.

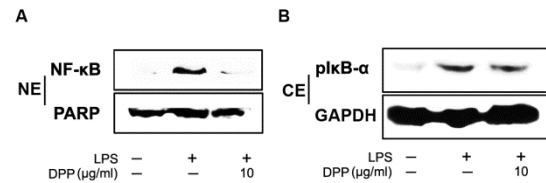


Fig. 4. DPP relieved the NF-κB activation in the activated RAW264.7 cells. Cells were pretreated with DPP for 1 h and then stimulated with LPS (10 μg/ml) for 2 h. The protein levels of (A) NF-κB and (B) pIκB-α were analyzed by Western blotting. PARP was used as a nuclear marker and a loading control. GAPDH was used as a loading control. LPS, lipopolysaccharide; DPP, Denmark porcine placenta; NE, nuclear extract; CE, cytoplasmic extract.

viability was evaluated using the MTT method. At doses from 0.1 μg/ml to 10 μg/ml, DPP did not inhibit the cell viability of RAW264.7 cells after 24 h incubation with LPS (Fig. 1D). DPP alone had no effect on inflammatory cytokine secretion and cell viability (data not shown).

DPP relieved the caspase-1 activity in the activated RAW264.7 cells

Due to caspase-1 playing important role in inflammatory regulation, we therefore also evaluated the influence of DPP on caspase-1 activities. The results showed that stimulation with LPS significantly elevated the caspase-1 activities compared to untreated cells (Fig. 2A, $p < 0.05$). However, DPP significantly suppressed the caspase-1 activities in a dose dependent manner (Fig. 2A, $p < 0.05$). Western blot analysis showed greatly decreased inactive caspase-1 protein levels in cells treated with LPS alone (Fig. 2B). By contrast, DPP treatment exhibited an increase of inactive caspase-1 protein levels (Fig. 2B).

DPP relieved the secretion of NO and expression of iNOS in the activated RAW264.7 cells

Next, we determined the influence of DPP on NO secretion, RAW264.7 cells were treated with DPP for 48 h. As shown in Fig. 3A, cells treated with LPS alone dramatically elevated the NO secretion compared to the untreated cells. However, DPP significantly suppressed the LPS-induced NO secretion (Fig. 3A, $p < 0.05$). In addition, we determined the influence of DPP on the iNOS expression. Consistent with the results of NO secretion, Western blotting revealed that DPP suppressed the iNOS expression in the activated RAW264.7 cells (Fig. 3B).

DPP relieved the NF-κB activation in the activated RAW264.7 cells

NF-κB, a transcription factor, is well known to control inflammation. In order to determine the role of the NF-κB during the pathogenesis of LPS-induced inflammation in vitro model, the expression of NF-κB were observed. Following LPS stimulation, NF-κB in the nuclear fractions was significantly enhanced when compared with untreated cells (Fig. 4A). By contrast, DPP significantly inhibited the LPS-stimulated NF-κB activation (Fig. 4A). In a parallel experiment, the expression levels of pIκB-α in the cytosolic compartment were determined. DPP treatment inhibited the expression of pIκB-α (Fig. 4B)

DISCUSSION

One plausible cause of fatigue is activation of the immune system characterized by general upregulation of proinflammatory cytokines. The general proinflammatory

cytokines are known to be caused by many diverse infectious and autoimmune diseases and have been implicated in disorders characterized by chronic fatigue (Buchwald et al., 1997; Giovannoni et al., 1997; Hahn et al., 1993). Evidence from clinical reports showed that administration of exogenous proinflammatory cytokines exacerbates symptoms of fatigue (Smedley et al., 1983). Bluthé et al., (2000) found that IL-1 tended to cause sickness behavior both in humans and animals. Another study reported a correlation between IL-1 β expression and fatigue-related behaviors (Ifuku et al., 2014). In addition, IL-6 levels were correlated with breast cancer patients experiencing fatigue (Bower et al., 2013). Injection of antibodies specific to either IL-1 or TNF improves fatigue symptoms in humans (Norheim et al., 2012; Omdal et al., 2005). Therefore, biological agents targeting proinflammatory cytokines has potential therapeutic advantages in treating fatigue. In the current study, DPP markedly suppressed the levels of these cytokines in FST fatigue mice and activated RAW264.7 cells. These results demonstrated that DPP exerts an anti-fatigue effect via the modulation of proinflammatory cytokines.

Induction of proinflammatory cytokine has been shown to mediate caspase-1 signaling pathway. Caspase-1 belongs to a large family of cysteine-aspartic acid protease and specifically cleaves proteins at an aspartic acid residue (Stutz et al., 2009). Caspase-1 preferentially cleaves the inactive IL-1 β which is critical component in orchestrating the inflammation (Stutz et al., 2009). Mice deficient for caspase-1 showed a decreased production of mature IL-1 β and was not able to induce inflammatory bowel disease (Li et al., 1995). Other reports underlining the importance of caspase-1 in a mouse model of fatigue with swim stress combined with LPS challenge, indicating that caspase-1 blocking therapies may be a treatment option for anti-fatigue and anti-inflammatory therapy (Zhang et al., 2016). In the current study, we found that DPP effectively suppressed both the catalytic activity and protein expressions of caspase-1. These results indicated that the anti-fatigue and anti-inflammatory properties of DPP may occur through regulation of caspase-1.

Chronic fatigue syndrome (CFS) is correlated with high levels of NO (Pall et al., 2000). The released IL-1, TNF- α , IL-6, and IFN- γ induce iNOS expression and subsequently, iNOS synthesizes NO. The increased levels of NO cause the tissue damage and some of the symptoms of CFS (Nijs et al., 2004; Pall et al., 2000). NF- κ B, a transcription molecule, required for NO secretion and regulated inflammatory gene expression (Libermann et al., 1990; Xie et al., 1994). Activation of cells by diverse of extracellular stimuli causes the phosphorylation and degradation of I κ B. Then, liberated NF- κ B reach the nucleus and interact with the κ B motif on the promoter region of target genes such as inflammatory cytokines and iNOS (Lenardo et al., 1989). Our results showed that DPP significantly decreased the secretion of NO by inhibiting iNOS expression and also effectively inhibited the NF- κ B activation. These results indicated that DPP exerts anti-fatigue and anti-inflammatory effects via controlling NF- κ B activation.

The placenta contains biologically active compounds, such as, proteins, hormones, growth factors, and peptides (Hong et al., 2015) and the diverse active molecules of placenta has anti-inflammatory properties. In particular, omega-3 fatty acids inhibited the expression of proinflammatory markers in mice (Skourliakou et al., 2010; Tieset et al., 2009). Furthermore, diverse amino acids, including glycine, cysteine, proline, and glutamic acids significantly ameliorated inflammatory disorders (Han et al., 2015; Kim MH et al., 2016; Nam et al., 2016) Therefore, the anti-inflammatory effects of DPP in the

present study might be exerted by components of placenta extracts, including fatty acids and amino acids.

In conclusion, our study demonstrated that DPP suppressed the inflammatory mediators via attenuating the caspase-1 and NF- κ B signaling pathways. Based on these findings, DPP could be useful in fatigue treatment as a potential anti-inflammatory agent. However, clinical study will be needed to demonstrate the anti-fatigue effects of the placenta.

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None.

CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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