Original Research

Effect of fermented porcine placenta on physical fatigue in mice

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Abstract

The fatigue spreads among the people who live under stressful life and brings about a negative impact on physical function. Here we evaluated the anti-fatigue effects of fermented porcine placenta (FPP) and main constituents, lysine (Lys) and leucine (Leu) with treadmill stress test and forced swimming test (FST) in animal models. The mice were administrated with FPP, Lys, and Leu for 21 days. After treadmill exercise, FPP, Lys, and Leu significantly reduced fatigue-related biochemical parameters, including lactate, lactate dehydrogenase, glucose, creatine kinase, urea nitrogen, cortisol, and pro-inflammatory cytokines, whereas superoxide dismutase activity and glycogen levels were significantly increased by FPP, Lys, and Leu. In the FST, FPP, Lys, and Leu significantly decreased immobility times and up-regulated brain-derived neurotrophic factor expression in brain. Furthermore, FPP, Lys, and Leu significantly decreased production of tumor necrosis factor-α, interleukin (IL)-6, IL-1β, and IL-4 through blockade of caspase-1/nuclear factor-κB pathway in stimulated splenocytes. In addition, FPP, Lys, and Leu significantly promoted proliferation of splenocytes. In conclusion, these findings suggest the potential of FPP as a novel functional food for the regulation of fatigue.

Keywords: Fermented porcine placenta, lysine, leucine, fatigue, biochemical parameters, treadmill test

Introduction

Fatigue is one of the most common and disabling non-motor problems and brings about a negative impact on physical function.[1] Fatigue spreads among the people who live under stressful life and can be seriously aggravated by the excessive stress.[1] In addition, dysfunction of the immune, endocrine, or antioxidant systems can lead to fatigue or fatigue-related disorders.[2] Numerous biochemical parameters, including lactate, glycogen, and blood urea nitrogen (BUN) are involved in fatigue.[3] Lactate dehydrogenase (LDH) and creatine kinase (CK) are precise indicators of muscle damage.[4] Fatigue is also associated with immune abnormalities in oxidative stress pathways or multiple inflammatory pathways.[5] Oxidative stress resulting from excessive exercise induces the production of excess free radicals that contribute intensively to fatigue.[6] Nitric oxide (NO) metabolites showed a much higher increase in chronic fatigue syndrome (CFS) patients.[7] Intracellular defense systems which consist of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) can eliminate free radicals.[5] In addition, the symptoms of fatigue are consistent with inflammatory cytokine dysregulation.[8]

Seriously, pharmacological drugs for treating fatigue cannot yet satisfy the need of the people. In our earlier study, porcine placenta extract regulated malnutrition-induced fatigue by decreasing fatigue-related factors.[9] Also, it was reported that the anti-inflammatory property of human placenta extract ameliorated chronic inflammation in CFS.[10] Scientific evidences demonstrated the numerous health benefits of placenta extract including immune modulation, depression, menopause, and cellular regeneration.[11–13] Fermentation is known to change protein content and enhances health benefits.[14] Also, fermentation can be considered as an effective process for increasing anti-fatigue effects.[15] Mitsui *et al.*[16] reported that fermented porcine placenta (FPP) is safe and nontoxic. Based on previous reports, we hypothesized that FPP and its constituents, lysine (Lys) and leucine (Leu) increased by fermentation could be preferentially enhancing anti-fatigue effect (Table 1). Thus, we investigated the anti-fatigue effects of FPP, Lys, and Leu on

Table 1 Ingredient variations of porcine placenta by the fermentation process

	(%)												
	Lys	Leu	Arg	Ala	Pro	Thr	His	Iso	Tyr	Met	Phe	Try	Val
PP	1.79	2.45	1.67	1.45	0.61	0.95	0.99	0.94	0.93	0.48	1.31	0.38	1.40
FPP	2.12	3.02	1.94	1.46	0.70	1.02	1.16	0.99	1.18	0.64	1.63	0.48	1.62

PP: porcine placenta, FPP: fermented porcine placenta, Lys: lysine, Leu: leucine, Arg: arginine, Ala: alanine, Pro: proline, Thr: threonine, His: histidine, Iso: isoleucine, Tyr: tyrosine, Met: methionine, Phe: phenylalanine, Try: tryptophane, Val: valine.

exercise-induced fatigue mice models, treadmill stress test and forced swimming test (FST) which are the most useful screening tests for anti-fatigue drugs.[17] And we examined the effect and regulatory mechanism of FPP, Lys, and Leu on production of inflammatory cytokines in the stimulated splenocytes.

Materials and methods

Animals

The male ICR (4 weeks old) was obtained from the Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Republic of Korea). Mice were acclimated to individual cages during seven days in a room with controlled lighting (12 h light–dark cycle), humidity (50–60%), and temperature (20–23°C). Experimental procedures and animal care were approved by the Animal Care Committee of Kyung Hee University (KHUASP [SE]-15-080).

Preparation of FPP

Considering safety, stability, and effective utilization of standardized materials, we purchased the commercial FPP (Fermented Placenta Extract Powder A (K), HORUS Co., Ltd. Japan) which is currently used in Japan and Korea for clarifying the role of FPP on anti-fatigue activity. Fermentation conditions are as follows. Porcine placenta was derived from pathogen-free porcine placenta, which was partially hydrolyzed by protease. Then, yeast and black strap powder were added to initiate fermentation, which was later filtered through 2 μm filter and spray dried. FPP was dissolved in distilled water (DW) and doses of 0.1, 1, and 10 mg/kg were determined according to a previous report.[10]

Amino acids analysis

Amino acids were evaluated by using automatic amino acid analyzer (Sykam GmbH, Germany). FPP is made up of Leu (3.02%), Lys (2.12%), arginine (1.94%), valine (1.62%), phenylalanine (1.63%), alanine (1.46%), histidine (1.16%), threonine (1.02%), proline (0.70%), and so on. We investigated the anti-fatigue effect of FPP and its main constituents, Lys and Leu. Lys and Leu were purchased from Sigma Chemical Co. (St. Louis, MO). The Lys and Leu were dissolved in DW and doses of 10 mg/kg were determined according to previous reports.[18,19]

Treadmill stress test

The mice in the each group were forced to run on the treadmill for 30 min once a week during three weeks. The pattern of loaded exercise composed of forced running at a 10 m/min during the 10 min, in sequence 16 m/min during the 10 min, last 21 m/min during the 10 min. On the 21th day, the speeds used for determination of the exhaustion were 10 m/min for 5 min, in sequence for 3 min each, then 40 m/min during the 30 min.[20] The administration of FPP (0.1, 1, and 10 mg/kg), Lys (10 mg/kg), and Leu (10 mg/kg) was continued for 21 days at the same time. Administration of DW was continued for 21 days at the same time in control group.

Forced swimming test

Based on the first measurement of immobility times, the mice were separated as control, FPP (0.1, 1, and 10 mg/kg), Lys (10 mg/kg), and Leu (10 mg/kg) groups. FPP (0.1, 1, and 10 mg/kg), Lys (10 mg/kg), and Leu (10 mg/kg) groups were orally administered to mice once per day for 21 days. Administration of DW was continued for 21 days at the same time in control group. The FST proceed at the end of administration period for the 21 days. The FST was performed as described previously.[21]

Fatigue-related biochemical parameters analysis in serum, muscle, or liver

After blood samples were collected from the heart in mice, the serum was separated. Muscle and liver were collected rapidly under standard conditions. The levels of lactate (#ab65331), LDH (#ab102526), glucose (#ab65333), CK (#ab155901), SOD (#ab65354), glycogen (#ab65620), and catalase (#ab83464) were determined using commercially available kits (Abcam, Cambridge, UK). The level of BUN (#K024-H1) was determined using commercially available kit (Arbor Assays, Ann Arbor, MI). The level of cortisol (#SE120082) was determined using commercially available kit (Sigma Chemical Co).

L6 cell culture

The rat skeletal muscle cell line (L6) was purchased from Korean Cell Line Bank and was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in 5% CO_2 with 95% atmosphere.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

L6 cells (1×10^4) or splenocytes cells (2×10^5) treated with FPP (0.1, 1, and 10 μg/mL), Lys (1 and 10 μg/mL), and Leu (1 and 10 μg/mL) for 24 h were incubated with MTT solution (5 mg/mL, Sigma Chemical Co.) for 4 h at 37°C in a 5% CO_2 with 95% atmosphere. Dimethyl sulfoxide was then added to the formazan and absorbance was measured at 540 nm using an ELISA reader.

Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines in serum, spleen, and splenocytes were measured by ELISA, as previously performed.[22] The levels of cytokines in spleen were divided according to the total protein levels which were measured using a bicinchoninic acid (BCA) protein assay kit (Sigma Chemical Co.).

Measurement of NO concentration

NO concentration in serum were measured by the Griess method.[22]

Isolation of splenocytes

The spleen was teased into a single cell suspension in RPMI-1640 with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mmol/L l-glutamine. The splenocytes cells (2×10^5) were treated with FPP (0.1, 1, and 10 μg/mL), Lys (1 and 10 μg/mL), and Leu (1 and 10 μg/mL) for each times.

Western blot analysis

Western blot was fulfilled on the extract of brain tissue or splenocytes, as previously performed.[20]

Nuclear protein extraction

Harvested splenocytes cells (3×10^5) were resuspended in 400 μL of ice-cold hypotonic buffer (0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 2 mmol/L $MgCl_2$, 10 mmol/L Hepes/KOH, 0.5 mmol/L phenylmethane sulfonyl fluoride (PMSF), 1 mmol/L dithiothreitol (DTT), and 10 mmol/L KCl, pH 7.9). After placing it on the ice for 10 min, it was vortexed and centrifuged at 15,000 g for 30 s. Supernatant aliquots including the cytoplasmic proteins were stored at −70°C. Pelleted nuclei were gently resuspended in 50 μL of ice-cold saline buffer (0.1 mmol/L EDTA, 50 mmol/L Hepes/KOH, 0.5 mmol/L PMSF, 1 mmol/L DTT, 50 mmol/L KCl, 300 mmol/L NaCl, and 10% glycerol, pH 7.9). After placing it on ice for 20 min, it was vortexed and centrifuged at 15,000 g for 5 min at 4°C. Supernatant aliquots including the nuclear proteins were stored at −70°C. Each protein level was estimated using a BCA protein assay method (Sigma, St. Louis, MO).

Assessment of proliferation

The proliferation of splenocytes was measured using a colorimetric immunoassay based on the bromodeoxyuridine (BrdU) incorporated measurement through the DNA synthesis (#11 647 229 001, Roche Diagnostics GmbH, Mannheim, Germany).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from splenocytes using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea) and total RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). We performed the quantitative real-time PCR using an SYBR Green master mix, and mRNA was detected using an ABI StepOne real time PCR System (Applied Biosystems, Foster City, CA). We used the following primers: mouse KI-67 (5′ CTT CAC TCT TAC TTT CCA CA 3′; 5′AAC ACC TAC AAA ATG ACT TC 3′) and mouse GAPDH (5′ TCG ACA GTC AGC CGC ATC TTC TTT 3′; 5′ ACC AAA TCC GTT GAC TCC GAC CTT 3′). Typical profile times used were as follows: initial step 95°C for 10 min followed by a second step at 95°C for 15 s and 60°C for 30 s for 40 cycles. Levels of target mRNAs were normalized versus GAPDH. Data were analyzed using the ΔΔCT method.

Statistical analysis

All data were checked for normality using the Shapiro-Wilk test. Treatment effects were analyzed using one-way analysis of variance (ANOVA) and the independent *t*-test. *In vitro* data are shown as mean ± standard error mean (SEM) from at least three independent experiments performed in duplicates or triplicates. *In vivo* data are represented as the mean ± SEM ($n = 5$/group). Statistical significance was accepted for P values < 0.05.
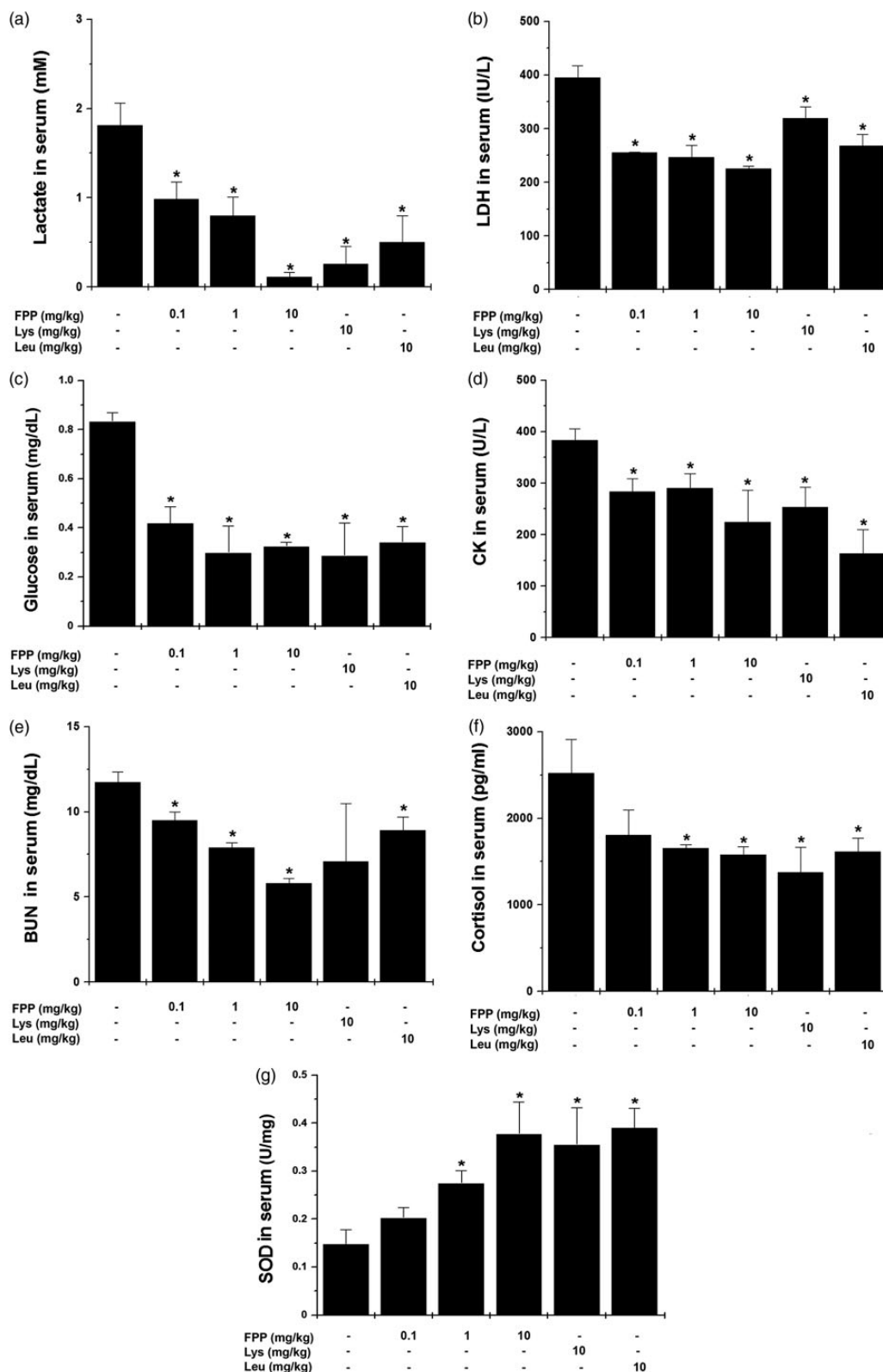
Results

Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in serum after treadmill stress test

First, we investigated whether FPP, Lys, and Leu would have an anti-fatigue effect after treadmill exercise by measuring blood biochemical parameters. As shown in Figure 1, administration of FPP and Leu down-regulated serum lactate, LDH, glucose, CK, BUN, and cortisol levels compared with the control group (Figure 1a to f, $P < 0.05$). Lys decreased serum lactate, LDH, glucose, CK, and cortisol levels after treadmill exercise (Figure 1a to f, $P < 0.05$). In addition, administration of FPP, Lys, and Leu up-regulated SOD levels in the serum compared with the control group (Figure 1g, $P < 0.05$).

Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in muscle after treadmill stress test

Next, we measured fatigue-related biochemical parameters in muscle of FPP, Lys, and Leu-treated mice. As shown in Figure 2, administration of FPP significantly down-regulated lactate, LDH, and CK levels and administration of Lys and Leu significantly down-regulated LDH and CK in muscle compared with the control

Figure 1 Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in serum after treadmill stress test. After the last treadmill exercise, serum samples were obtained from heart. (a) Lactate, (b) LDH, (c) glucose, (d) CK, (e) BUN, (f) cortisol, and (g) SOD were measured with each kit. Values are the mean $\pm$ SEM. $n = 5$ per group. BUN: blood urea nitrogen, CK: creatine kinase, FPP: fermented porcine placenta, LDH: lactate dehydrogenase, Lys: lysine, Leu: leucine, SEM: standard error mean, SOD: superoxide dismutase. *$P < 0.05$, significantly different from the control mice
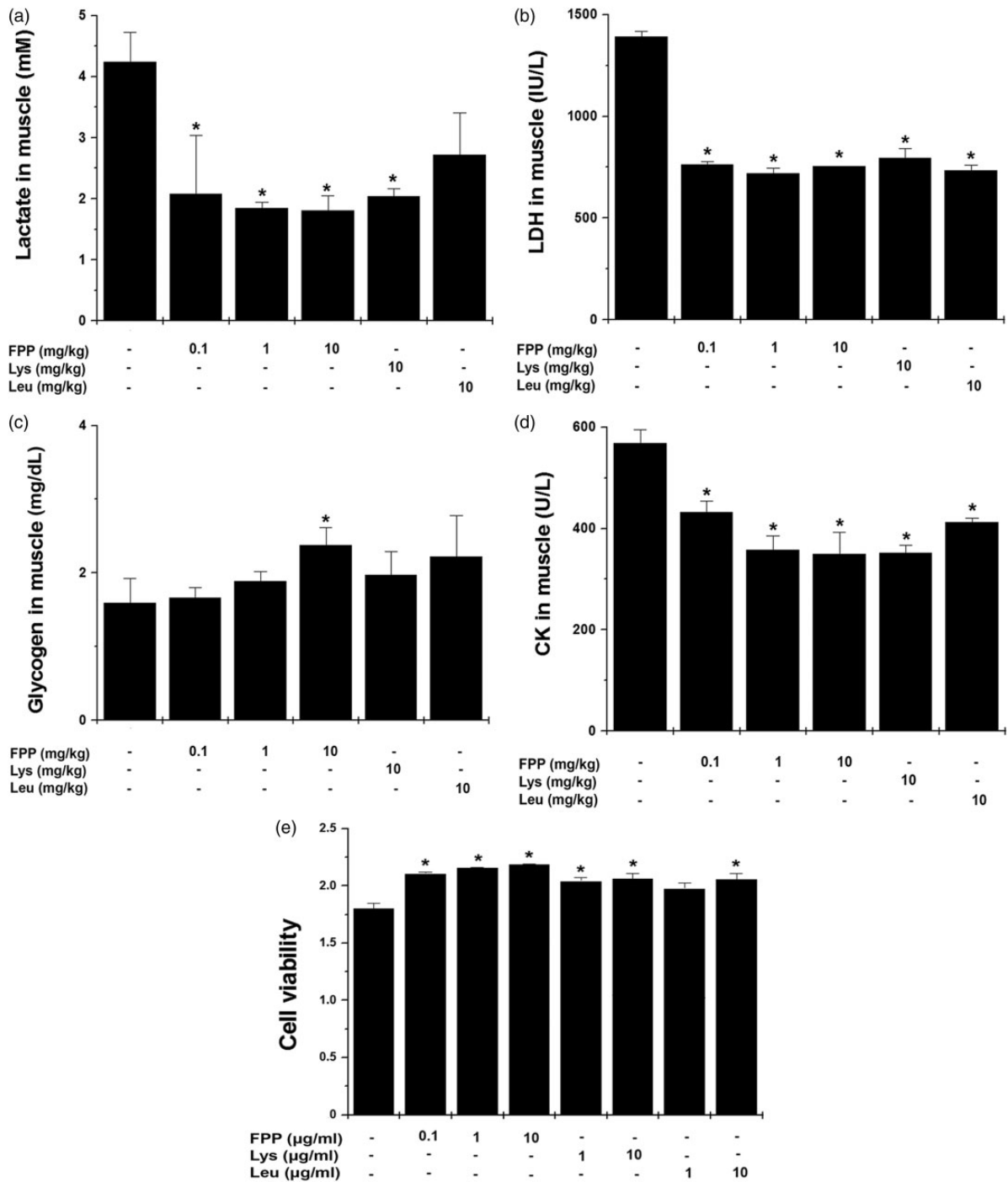
Figure 2 Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in muscle after treadmill stress test. After the last treadmill exercise, muscle samples were obtained from each mouse. (a) Lactate, (b) LDH, (c) glycogen, and (d) CK were measured with each kit. (e) L6 cells were treated with FPP, Lys, and Leu for 24 h except FBS. The viability of L6 cells was measured by a MTT assay. Values are the mean ± SEM. $n = 5$ per group. CK: creatine kinase, FBS: fetal bovine serum, FPP: fermented porcine placenta, LDH: lactate dehydrogenase, Lys: lysine, Leu: leucine, MTT: 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SEM: standard error mean. *$P < 0.05$, significantly different from the control mice

group (Figure 2a, b, and d, $P < 0.05$). By contrast, muscle glycogen content of FPP group was significantly higher than that of the control group (Figure 2c, $P < .05$). Further, we examined whether FPP, Lys, and Leu would regulate muscle damage *in vitro* model using L6 cells. FPP, Lys, and Leu significantly improved viability of L6 cells (Figure 2e, $P < 0.05$).

Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in liver after treadmill stress test

Liver is an important organ involved in long-term exercise. The prolonged exercise increases oxidative stress in the liver.[23] Administration of FPP, Lys, and Leu significantly up-regulated SOD and CAT levels in liver compared with

Table 2 Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters in liver and spleen after treadmill

		Control	FPP (0.1 mg/kg)	FPP (1 mg/kg)	FPP (10 mg/kg)	Lys (10 mg/kg)	Leu (10 mg/kg)
Liver	Glycogen	2.17 ± 0.15	$4.51 \pm 0.74^*$	$5.07 \pm 0.21^*$	$8.24 \pm 1.45^*$	$3.37 \pm 0.40^*$	$4.99 \pm 1.05^*$
	SOD	3.36 ± 0.41	$8.09 \pm 1.56^*$	$10.71 \pm 1.22^*$	$12.76 \pm 1.22^*$	$4.43 \pm 0.36^*$	$8.08 \pm 2.44^*$
	Catalase	1.30 ± 0.11	$2.48 \pm 0.58^*$	$2.66 \pm 0.41^*$	$4.12 \pm 0.90^*$	$1.70 \pm 0.25^*$	$2.52 \pm 0.55^*$
Spleen	TNF-α	0.34 ± 0.05	$0.14 \pm 0.09^*$	$0.14 \pm 0.09^*$	$0.06 \pm 0.13^*$	$0.18 \pm 0.06^*$	$0.15 \pm 0.06^*$
	IL-6	0.08 ± 0.01	$0.02 \pm 0.01^*$	$0.02 \pm 0.01^*$	$0.01 \pm 0.001^*$	$0.007 \pm 0.003^*$	$0.004 \pm 0.002^*$
	IL-1β	6.53 ± 0.79	$5.08 \pm 1.66^*$	$3.63 \pm 0.97^*$	$1.06 \pm 0.16^*$	$4.58 \pm 0.96^*$	$3.24 \pm 0.60^*$
	IL-4	0.15 ± 0.09	$0.05 \pm 0.001^*$	$0.01 \pm 0.01^*$	$0.01 \pm 0.01^*$	$0.01 \pm 0.01^*$	$0.01 \pm 0.01^*$
	IFN-γ	1.76 ± 0.92	3.57 ± 1.75	4.27 ± 0.99	$8.90 \pm 2.16^*$	2.98 ± 0.57	1.94 ± 2.73

The administration of FPP, Lys, and Leu was continued for 21 days at the same time. After the last treadmill test, liver and spleen samples were obtained the from each mouse. Glycogen (mg/dL), SOD (U/mg), and catalase (mU/mL) were measured by the each assay kit. The levels of TNF-α, IL-6, IL-1β, IL-4, and IFN-γ were divided according to total protein levels and determined by the ELISA method. Values are the mean $\pm$ SEM. $n = 5$ per group. FPP: fermented porcine placenta, IFN: interferon, IL: interleukin, Lys: lysine, Leu: leucine, SOD: superoxide dismutase, TNF: tumor necrosis factor.
$^*P < 0.05$, significantly different from the control mice.

the control group (Table 2, $P < 0.05$). Glycogen was also significantly up-regulated by FPP, Lys, and Leu compared with the control group (Table 2, $P < 0.05$).

Effect of FPP, Lys, and Leu on fatigue-related cytokines in serum and spleen after treadmill stress test

We studied whether FPP, Lys, and Leu would regulate the levels of inflammatory cytokines in serum after treadmill exercise. Administration of FPP significantly down-regulated levels of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-1β, IL-4, and NO in serum compared with the control group (Figure 3a to d and f, $P < 0.05$,). Also, FPP significantly up-regulated serum interferon (IFN)-γ level compared with the control group (Figure 3e, $P < 0.05$). Lys significantly down-regulated levels of serum TNF-α, IL-6, IL-1β, and NO, whereas it up-regulated serum IFN-γ level compared with the control group (Figure 3a to f, $P < 0.05$). Leu also significantly decreased levels of serum IL-6, IL-1β, and NO, whereas it up-regulated serum IFN-γ level compared with the control group (Figure 3b to f, $P < 0.05$). In spleen, administration of FPP, Lys, and Leu significantly down-regulated the levels of inflammatory cytokines, including TNF-α, IL-6, IL-1β, and IL-4 compared with the control group (Table 2, $P < 0.05$). FPP significantly up-regulated IFN-γ levels in spleen compared with the control group (Table 2, $P < 0.05$).

Effect of FPP, Lys, and Leu on FST

Furthermore, we investigated an anti-fatigue effect of FPP, Lys, and Leu by performing another fatigue model, FST. The immobility times of the mice administrated with FPP, Lys, and Leu were significantly down-regulated compared with the control group at 21 days (Figure 4a, $P < 0.05$). Figure 4 shows that FPP, Lys, and Leu significantly down-regulated serum lactate level compared with the control group (Figure 4b, $P < 0.05$). Lys significantly decreased serum LDH level compared with the control group (Figure 4c, $P < 0.05$). Leu significantly reduced glucose level compared with the control group (Figure 4d, $P < 0.05$). However, FPP did not significantly decrease the

levels of LDH and glucose. In brain, brain-derived neurotrophic factor (BDNF) expression was also up-regulated by FPP, Lys, and Leu (Figure 4e).

Effect of FPP, Lys, and Leu on fatigue-related cytokines in splenocytes

Given up-regulation of inflammatory cytokines levels in spleen after treadmill exercising, we investigated the mechanism of an anti-fatigue effect of FPP, Lys, and Leu in anti-CD3 and anti-CD28 antibodies-stimulated splenocytes. Treatment with FPP, Lys, and Leu significantly down-regulated the production of TNF-α, IL-6, IL-1β, and IL-4 from anti-CD3 and anti-CD28 antibodies-stimulated splenocytes (Figure 5a, $P < 0.05$,). It is reported that the caspase-1 regulates the production of TNF-α, IL-6, and IL-1β.[24] Thus, we examined the regulatory effects of FPP, Lys, and Leu on the caspase-1 activity. FPP, Lys, and Leu significantly decreased caspase-1 activity in activated splenocytes (Figure 5b, $P < 0.05$). Also, FPP, Lys, and Leu significantly decreased caspase-1 protein levels in activated splenocytes (Figure 5c, $P < 0.05$). Nuclear factor (NF)-κB is a crucial transcription factor required for the pro-inflammatory cytokines expression, such as TNF-α, IL-6, and IL-1β.[25] Thus, we performed the regulatory effects of FPP, Lys, and Leu on the translocation into nucleus of NF-κB and phosphorylation of IκBα. As shown in Figure 5(d), FPP, Lys, and Leu inhibited level of NF-κB in nucleus and phosphorylation of IκBα in cytosol of activated splenocytes ($P < 0.05$).

Effect of FPP, Lys, and Leu on proliferation of splenocytes

Finally, we evaluated proliferation markers, such as BrdU incorporation and KI-67 mRNA expression to investigate the effects on the immunity enhancement of FPP, Lys, and Leu *in vitro*. Treatment with FPP, Lys, and Leu significantly promoted BrdU incorporation in anti-CD3 and anti-CD28 antibodies-stimulated splenocytes (Figure 6a, $P < 0.05$). Also, FPP, Lys, and Leu significantly up-regulated KI-67 mRNA expression in stimulated splenocytes (Figure 6b,
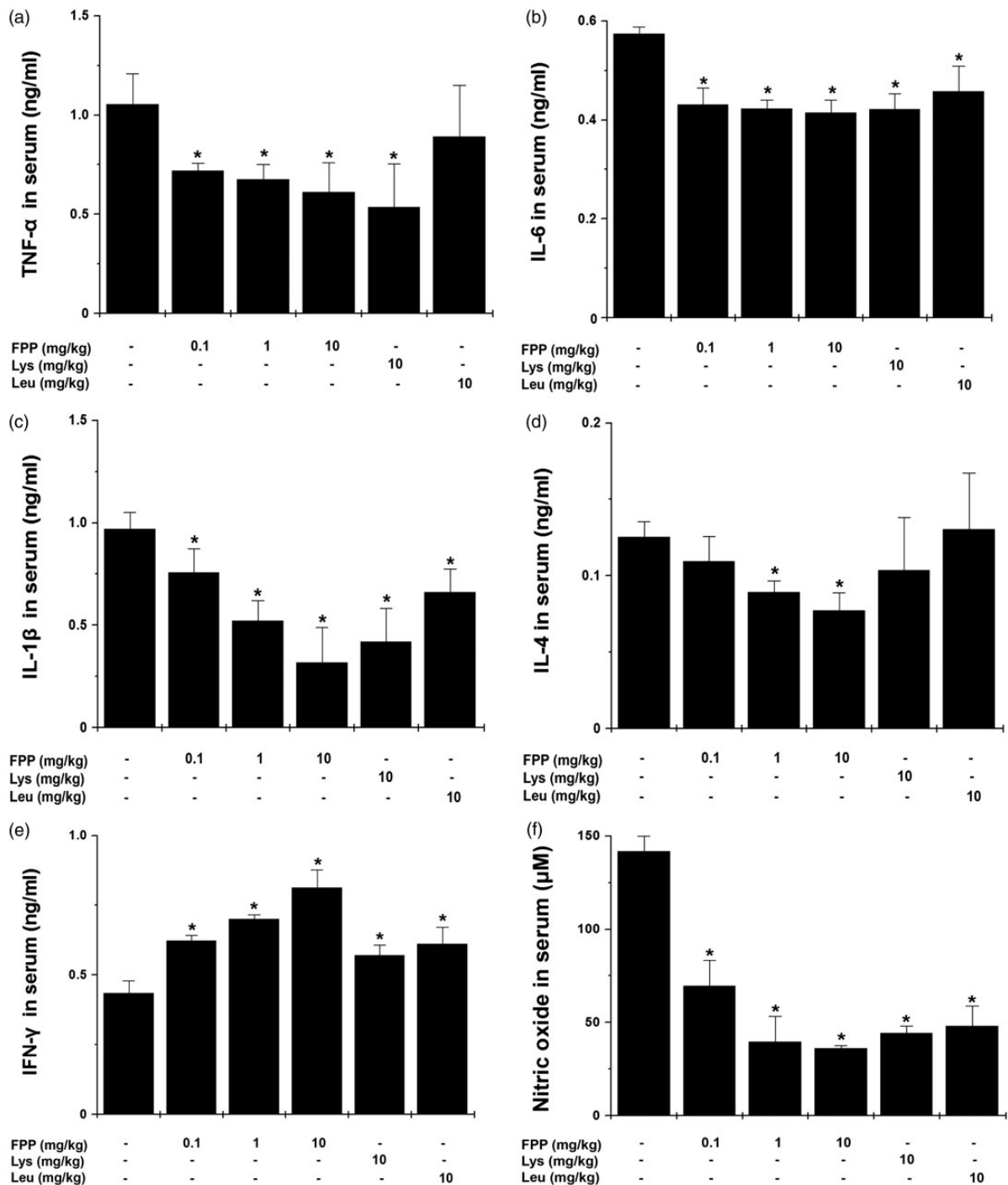
Figure 3 Effect of FPP, Lys, and Leu on fatigue-related cytokines in serum after treadmill stress test. After the last treadmill exercise, serum sample were obtained from heart. The levels of (a) TNF-α, (b) IL-6, (c) IL-1β, (d) IL-4, and (e) IFN-γ were analyzed by the ELISA method. (f) The levels of nitric oxide were measured by the Griess method. Values are the mean ± SEM. $n = 5$ per group. ELISA: enzyme-linked immunosorbent assay, FPP: fermented porcine placenta, IFN: interferon, IL: interleukin, Lys: lysine, Leu: leucine, SEM, standard error mean, TNF: tumor necrosis factor. *$P < 0.05$, significantly different from the control mice

$P < 0.05$). Cytotoxicity was not shown in all instances (Figure 6c).

Discussion

In our research, we observed the anti-fatigue effect of FPP, Lys, and Leu on several biochemical markers in treadmill stress test and FST *in vivo* models and splenocytes *in vitro* model.

Exercise causes accumulation and production of metabolic products, such as lactate.[26] Serum lactate and LDH are increased in exercise-induced fatigue model.[27] An increase in CK with LDH is index of cellular necrosis and tissue damage in skeletal muscles.[28] Because most of the CK in
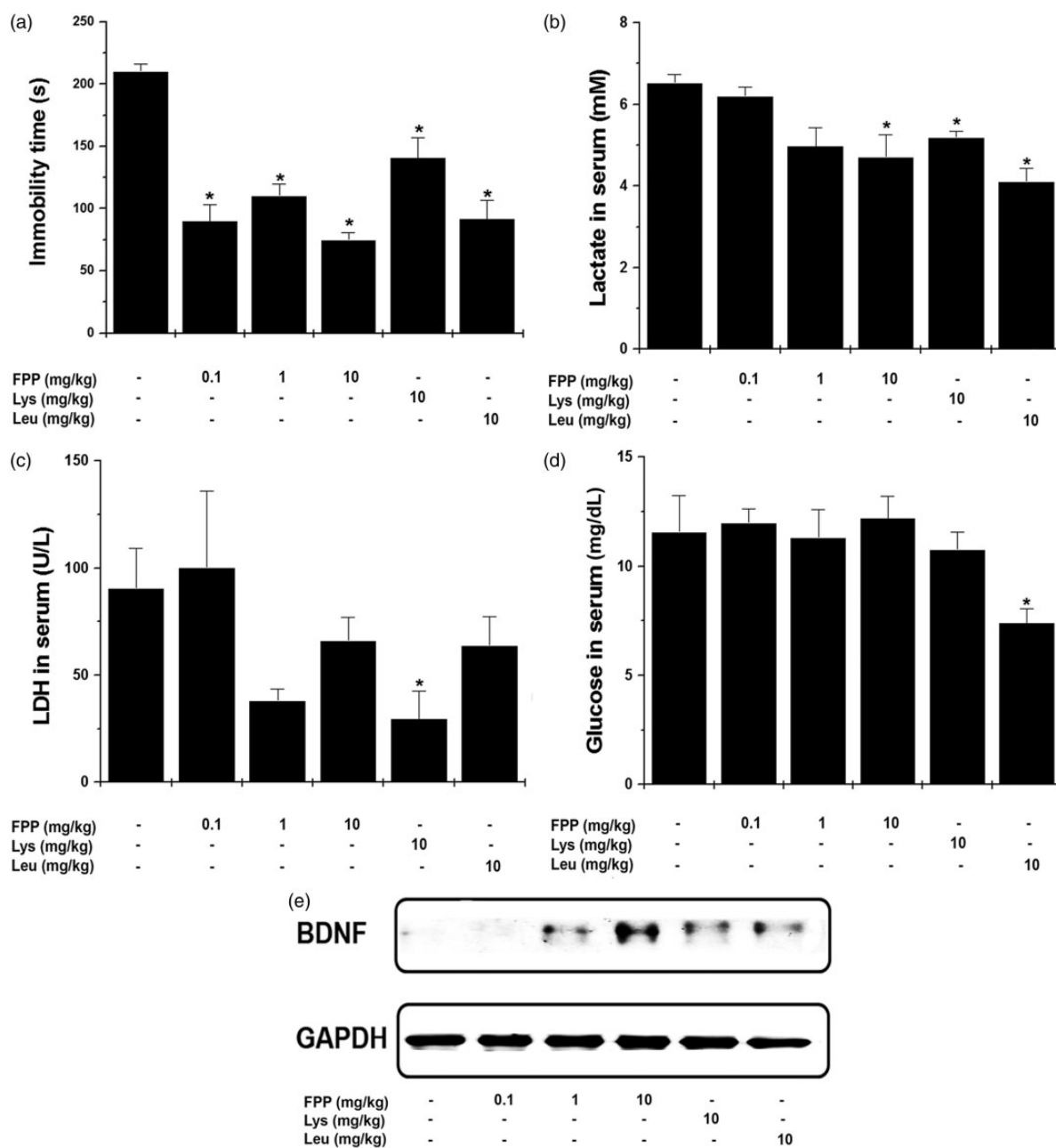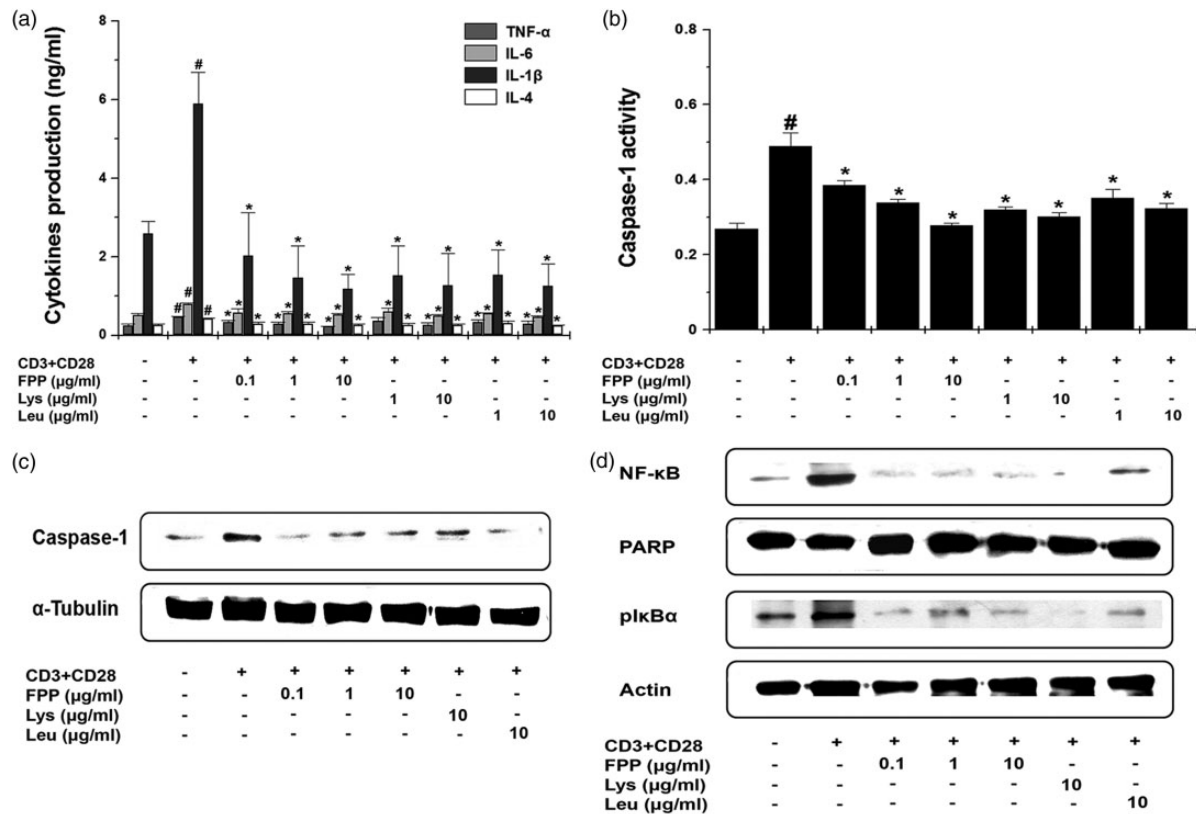
Figure 4 Effect of FPP, Lys, and Leu on fatigue-related biochemical parameters after FST. (a) The immobility times of the mice were measured at the 21st days. (b–d) After the FST, serum samples were obtained from heart. The serum lactate and LDH levels were measured with each kit. (e) The protein levels of BDNF in brain were analyzed by the Western blotting. Values are the mean $\pm$ SEM. $n = 5$ per group. BDNF: brain-derived neurotrophic factor; FPP: fermented porcine placenta, FST: forced swimming test, LDH: lactate dehydrogenase, Lys: lysine, Leu: leucine; SEM: standard error mean. *$P < 0.05$, significantly different from the control mice

the body is generally in the muscle, an increase in CK in blood shows muscle damage.[4] A positive correlation is between BUN and exercise tolerance.[29] The significant increases in BUN, LDH, and CK were observed in the specimens obtained after the marathon compared with the pre-marathon specimens.[30] Our results show that the levels of lactate, LDH, CK, and BUN in FPP or Leu-treated mice were lower than those in the control group on treadmill stress test and lactate levels in FPP, Lys, and Leu-treated mice were lower than those in the control group on FST. In addition, FPP, Lys, and Leu increased the viability of L6 cells,

suggesting that they ameliorate muscle damage. Thus, we suggest that FPP may have anti-fatigue effect through down-regulating the accumulation of metabolites in exercise-induced fatigue.

Cortisol is known as a biomarker of stress from physical or psychological stimuli. Serum cortisol measurement has been widely performed to evaluate physical stress response to strenuous exercise.[31] In stressful situations, cortisol can provide glucose to the body and leads to high blood sugar levels.[32] *Schisandra chinensis* reduced levels of serum cortisol and glucose in rats under strenuous swimming exercise and

Figure 5 Effect of FPP, Lys, and Leu on fatigue-related cytokines in splenocytes. (a) Splenocytes (2×10^5) were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies and treated with FPP, Lys, and Leu for 24 h. The production of TNF-α, IL-6, IL-1β, and IL-4 was analyzed by the ELISA method. (b) Splenocytes (2×10^5) were stimulated and treated with FPP, Lys, and Leu for 24 h. Caspase-1 activity was measured by a caspase-1 assay kit. Results show the mean ± SEM of data from three separate experiments with triplicate samples. (c) Splenocytes (2×10^5) were stimulated and treated with FPP, Lys, and Leu for 45 min. Caspase-1 protein levels were analyzed by the Western blotting. (d) Splenocytes (2×10^5) were stimulated and treated with FPP, Lys, and Leu for 45 min. The level of NF-κB in nuclear extract and level of phosphorylated IκBα in cytoplasmic extract were analyzed by the Western blotting. ELISA: enzyme-linked immunosorbent assay, FPP: fermented porcine placenta, IL: interleukin, Lys: lysine, Leu: leucine, NF: nuclear factor, pIκBα: phosphorylation of IκBα, SEM: standard error mean, TNF: tumor necrosis factor. [#]$P < 0.05$, significantly different from the un-stimulated splenocytes. *$P < 0.05$, significantly different from the stimulated splenocytes

decreased the stress.[33] Our result also shows that cortisol and glucose levels increased by treadmill exercise were inhibited by FPP, Lys, and Leu. Thus, we speculate that FPP, Lys, and Leu might reduce extreme exercise-induced stress, indirectly decreasing stress-induced high blood glucose levels. The main repositories of glycogen which is the polymeric storage form of glucose are skeletal muscle and liver.[34] Glycogen is a critical source of energy for skeletal muscle contraction in prolonged exercise.[35] During excessive exercise, the depletion of glycogen severely restricted energy supply.[36] However, protein ingestion alleviated the fatigue caused by the depletion of muscle glycogen during exercise endurance.[36] Our results show that administration of FPP after treadmill exercise increased glycogen level in muscle and liver. Thus, we suppose that administration of FPP would protect skeletal muscle and liver from glycogen depletion. And this result would indicate that FPP, Lys, and Leu have an anti-fatigue effect, particularly regulating fatigue-related factors in muscle and liver. Thus, we assume that FPP-treated mice might not need more energy during prolonged exercise than control mice.

The betterment of antioxidant enzyme activities can ameliorate fatigue because the antioxidant defense gets weaker during fatigue.[6] The negative association was found between serum NO and fatigue.[37] In addition, the antioxidants have considered as anti-fatigue materials by increasing SOD or CAT levels in liver.[23] Our results show that FPP, Lys, and Leu increased SOD and CAT levels and decreased NO level after treadmill exercise. We speculate that FPP, Lys, and Leu would improve the antioxidant defense of the mice after extensive exercise and confer anti-fatigue capability.

In fatigue, BDNF is a kind of neurotrophic factors which play a critical role in brain development, memory, and cognitive performance and known to be an attractive therapeutic target for chronic fatigue.[38] A decrease in BDNF expression induces stress in fatigue.[39] Our results show that FPP, Lys, and Leu up-regulated the BDNF levels in brain. Therefore, this result indicates that FPP, Lys, and Leu can down-regulated the fatigue through up-regulating the BDNF levels in brain.

Strenuous exercise induces the secretion of the inflammatory cytokines in muscle.[40] Pro-inflammatory cytokines were produced by intense physical stress in muscle after strenuous exercise.[8] The levels of TNF-α, IL-6, and IL-1β were increased in skeletal muscle and serum in patients
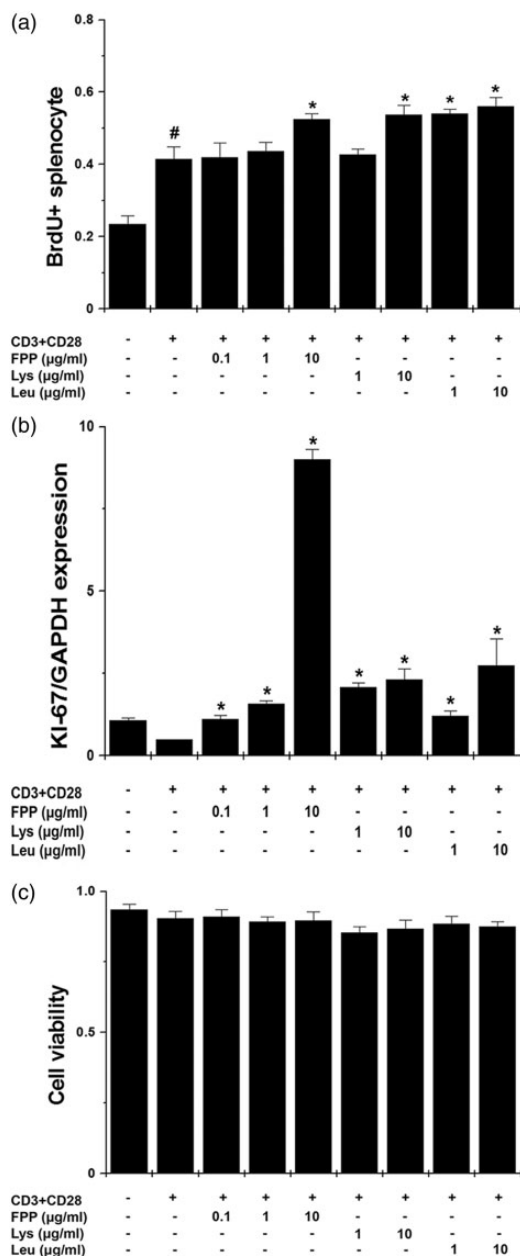
Figure 6 Effect of FPP, Lys, and Leu on proliferation of splenocytes. (a) Splenocytes (2×10^5) were stimulated with immobilized anti-CD3 and soluble anti-CD28 antibodies and treated with FPP, Lys, and Leu for 48 h. BrdU incorporation assay was performed. (b) The mRNA levels of KI-67 were measured by the quantitative real-time PCR. (c) The cytotoxicity was measured by a MTT assay. Results show the mean ± SEM of data from three separate experiments with triplicate samples. FPP: fermented porcine placenta, Lys: lysine, Leu: leucine, MTT: 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PCR, polymerase chain reaction, SEM: standard error mean. $^{\#}P < 0.05$, significantly different from the un-stimulated splenocytes. $^{*}P < 0.05$, significantly different from the stimulated splenocytes

with CFS.[3,41] The production of pro-inflammatory cytokines was suppressed through blockade of caspase-1 and NF-κB/IκBα pathway in splenocytes.[42] Our results show that FPP, Lys, and Leu down-regulated the production of TNF-α, IL-6, IL-1β, and IL-4 as well as the activation of caspase-1 and NF-κB in stimulated splenocyte. Interestingly, FPP, Lys, and Leu up-regulated the production of IFN-γ from stimulated splenocyte. And FPP, Lys, and Leu increased IFN-γ levels in serum and spleen after treadmill exercise. IFN-γ on un-stimulated T cells enhances adaptive immunity by augmenting their survival during the immune response.[43] An increase in INF-γ accelerated muscle healing after skeletal muscle injury.[44] Thus, we suppose that FPP, Lys, and Leu would have anti-fatigue activity, improving inflammatory reaction through the blockade caspase-1/NF-κB and enhancing immunity by increasing IFN-γ.

Proliferation of naïve T cells from the spleen is promoted in response to anti-CD3 antibody or anti-CD28 antibody.[45] T lymphocyte proliferation is associated with stimulation of immune system.[46,47] Arginine enhanced the splenocyte proliferation and immune function in an animal model.[48] In this study, FPP, Lys, and Leu promoted the splenocyte proliferation without inducing cytotoxicity. Thus, FPP, Lys, and Leu might possess anti-fatigue activity by enhancing immune responses through regulating T lymphocyte proliferation.

Nutritional deficits can be a factor inducing fatigue.[49] The porcine placenta contains many prime nutrients regulating stress, cellular protection, and immune function.[17] Fermentation has been used to produce more organic acids, amino acids, and vitamins in food or feed applications.[50] Lys and Leu contents were the highest among amino acids during the fermentation process of porcine placenta (Table 1). Stress-induced fatigue was associated with a decrease in Lys in blood.[51] Leu administration inhibited inflammatory responses and muscle soreness induced by severe exercise.[52] In addition, Hou et al.[53] reported that fermented placenta inducing bioactive peptides can be developed as ingredients in functional foods and dietary supplements with specific health benefits. Interestingly, porcine placenta extract (0.1 and 1 mg/kg) did not significantly inhibit the immobility times in our previous study,[9] whereas in this study, FPP (0.1, 1, and 10 mg/kg) significantly inhibited immobility times. Thus, we suggested that FPP was more effective than porcine placenta extract. However, it is necessary to comparative study about anti-fatigue effects between FPP and porcine placenta extracts.

In conclusion, the present study shows that supplementation with FPP, Lys, and Leu ameliorated fatigue by modulation of fatigue related-biochemical parameters. Therefore, this study signifies the potential of FPP toward anti-fatigue. This study is the first to explore pharmacological effects of FPP on fatigue using treadmill stress and FST models, so further studies will be required to determine.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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