2019 4(2) 1-6 Submission: 2019/11/10, Peer review: 2019/11/20, Accepted: 2019/11/25, Published: 2019/12/30

Kinesiology

Publication state: Japan
ISSN: 2435-0702

Publisher: J-INSTITUTE
Website: http://www.j-institute.jp

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http://dx.doi.org/10.22471/kinesiology.2019.4.2.01

A SPORTS Nutrition Supplement, Albizia Julibrissin Extract Fermented with Kefir Yogut Regulates Lipopolysaccharideinduced Inflammatory Signal

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Abstract

Although the inflammatory response is vital for biological regulation, chronic inflammation damages the human body. Presently available anti-inflammatory drugs, including steroids, have side effects that prevent their administration to athletes. This study aimed at elucidating a method to control exercise-induced inflammation and oxidative stress, by investigating the anti-inflammatory and antioxidant efficacy of extracts of Albizia julibrissin *fermented with kefir yogurt(FKA) in vitro. We investigated the effect of FKA on RAW 264.7 cells using cell viability assays. The regulatory effect of FKA in lipopolysaccharide(LPS)-induced nitric oxide(NO) production was determined using Griess reagent and the reverse transcription polymerase chain reaction(RT-PCR) assays, with specific primers such as interleukin(IL)-1β, IL-6, and IL-10, inducible nitric oxide synthase(iNOS), and cyclooxygenase- 2(COX-2) in vivo. Furthermore, the 2,2-diphenyl-1-picrylhydrazyl(DPPH) radical scavenging assay was performed and the cellular morphology changes were observed using florescence microscopy. The results showed that FKA significantly reduced LPS–induced NO production by regulating the expression of IL-1β, iNOS, and COX-2; thus, FKA exerts cytoprotective effects against ROS-induced cell death. These data suggest the anti-inflammatory therapeutic potential of FKA.*

[Keywords] ***Albizia Julibrissin, Kefir, Anti-Inflammation, IL-1β, iNOS***

1. This Regulation is Aimed at the Systemization

1.1. Introduction

Inflammation is a biological defense mechanism used by the body to protect against stimuli such as cytokines and free radicals, which occur during tissue injury or infection. When macrophages are stimulated by lipopolysaccharides (endotoxins), they release inflammatory cytokines such as interleukin(IL)-1β and IL-6, which induce the expression of nitric oxide synthase (iNOS), cyclooxygenase-2(COX-2), and inflammatory factors such as nitric oxide(NO) and Prostaglandin E2[1].

Although NO generation plays a crucial role in cellular immune homeostasis during bacterial infections, its excessive generation adversely affects cellular metabolism. Particularly, the binding of NO to reactive oxygen species(ROS) during exercise can cause muscle ache and damage, which can be fatal to athletes[2][3].

Kefir is mushroom-shaped fermented milk made by Tibetan monks for immunity enhancement, and is well known as Tibetan mushroom yogurt. It is fermented using yeast, and produces vitamins, essential amino acids, and polysaccharides, which augment its bioactive function by improving its antimicrobial effect[4].

Albizia julibrissin has been used in Korean traditional medicine to improve muscle pain and stamina[5]. In the present study, we investigated the effects of *Albizia julibrissin* extract fermented with kefir yogurt(FKA) on lipopolysaccharide(LPS)-stimulated RAW 264.7 macrophages.

2. Methods

2.1. Chemicals

Cell culture reagents were purchased from Gibco-BRL(Gaithersburg, MD, USA). The EZ-cytox cell viability assay was obtained from Daeil Lab Service(Seoul, Korea). The LIVE/DEAD cell viability assay kit was purchased from Thermo Fisher Scientific(Grand Island, NY, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Experimental design

The EZ-cytox cell viability assay was used to evaluate cell viability, the reverse transcription polymerase chain reaction(RT-PCR) to test the mRNA expression levels of cytokines and inflammatory factors, and the ROS generation assay to determine the cytoprotective effect of FKA.

2.3. Procedures

2.3.1. Fermented plant extraction

Albizia julibrissin was obtained from Dongguk University Oriental Hospital, Korea. One hundred grams of the plant was blended, the crude powder fermented with Kefir yogurt at 25°C for 3 days, and then precipitated with 1000 mL of sterile deionized water at 100°C for 3 h. The aqueous extracts were concentrated and evaporated at 60°C in a vacuum. The extract was then dissolved in 50 mL of sterile deionized water, and lyophilized by freeze-drying at -60°C.

2.3.2. Cell culture and cell viability assay

Cell culture and viability assay were performed as previously reported[6]. RAW 264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin, in a humidified atmosphere containing 5% CO_2 at 37°C. The cells(5×10^4 cells) were seeded in a 96-well microplate, and incubated with different concentrations of FKA for 24 h. Thereafter, cell viability was measured with ELISA using the EZ-cytox cell viability assay kit. The data were acquired using an ELISA reader according to the manufacturer's instructions.

2.3.3. NO generation assay

This assay was performed as previously reported [6]. RAW 264.7 cells in a 96-well microplate(5×10^4 cells/well) were co-treated with 10-30 μg/mL FKA, in the absence or presence of 100 ng/mL LPS, and cultured for 48 h in DMEM supplemented with 10% FBS. To determine the total concentration of NO in the culture media, Griess regent was added to 100 μL of the supernatant of each treatment condition, and absorbance was measured at 520 nm using a microplate reader.

2.3.4. RT-PCR

RT-PCR assays were performed as previously reported[7]. The total RNA in the cell lysates were isolated using TRI-reagent and then cDNA was synthesized using 0.5 μg of total RNA; the superscript II reverse transcription system with oligo-deoxythymidine. PCR amplification was performed using the following protocol: predenaturation at 95℃ for 3 min, and then 30 cycles of denaturation at 94℃ for 50 s, annealing at 55℃ for 50 s, and extension at 72℃ for 50 s, followed by a final extension at 72℃ for 10 min. The primers used were as follows: iNOS forward: 5'-ATG TCC GAA GCA AAC ATC AC-3'; iNOS reverse: 5'-TAA TGT CCA GGA AGT AGG TG-3'; COX-2 forward: 5'-GGA GAG ACT ATC AAG ATA GTG ATC-3'; COX-2 reverse: 5'-ATG GTC AGT AGA CTT TTA CAG CTC-3'; IL-1β forward: 5'- TTG ACG GAC CCC AAA AGA TG-3'; IL-1β reverse: 5'- AGA AGG TGC TCA TGT CCT CA-3'; IL-6 forward: 5'- CCA CTT CAC AAG TCG GAG GCT T-3'; IL-6 reverse: 5'- CCA GCT TAT CTG TTA GGA GA-3'; IL-10 forward: 5'- ACC TGGTAG AAG TGA TGC CCC AGG CA-3'; IL-10 reverse: 5'- CTA TGC AGT TGA TGA AGA TGT CAA A-3'; GAPDH forward: 5'- GCC CAT CAC CAT CTT CCA G-3'; GAPDH reverse: 5'- TGA GCC CTT CCA CAA TGC C-3'. The mRNA expression was quantified using an ethidium bromide-stained 1.5% agarose gel. The stained bands were visualized and analyzed using the Image J Software.

2.3.5. ROS radical scavenging analysis

The effect of FKA on DPPH radical scavenging was determined as previously reported [8]. Various concentrations of FKA were prepared using water, and 50 uL of FKA(W/v) mixed with 1 mL of DPPH ethanol solution(0.1 mM) and 450 uL of Tris-HCl buffer(50 mM, pH 7.4) was added. The mixture was then incubation for 1 h at 25°C, and the absorbance was measured at 517 nm. Cellular morphological changes were observed, and images were captured using an inverted microscope connected to a digital camera(IX71; Olympus, Tokyo, Japan).

2.3.6. Statistical analysis

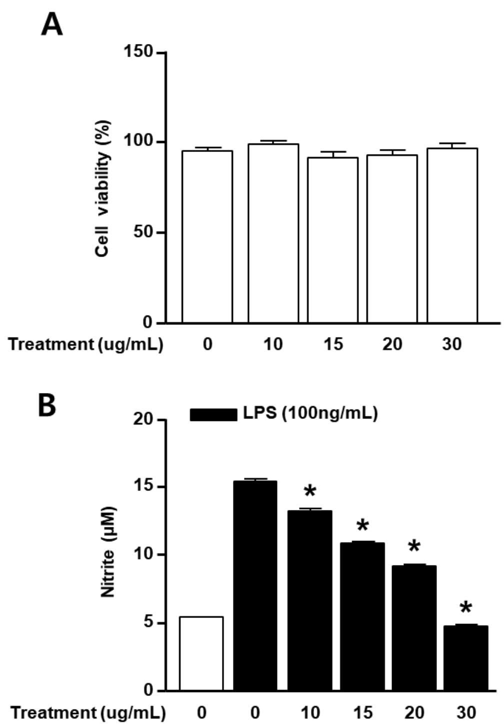
Data are expressed as the mean ± standard error of the mean(S.E.M.) of the indicated number of experiments. Statistical analysis was performed using the Student’s *t*-test for comparisons between 2 groups, using GraphPad prism(GraphPad Software, San Diego, CA, USA). p <0.05 was considered statically significant.

3. Results

3.1. Effect of FAK on cell viability and NO production in LPS-stimulated RAW 264.7 cells

We assessed whether FKA can regulate NO generation in LPS-induced RAW 264.7 cells. As shown in Figure 1A, FKA showed no cytotoxicity at doses up to 30 μg/mL. LPS-stimulated RAW 264.7 cells were treated with 10-30 μg/mL FKA for 24 h <Figure 1B>. LPS(100 ng/mL) significantly induced NO production, whereas FKA significantly reduced NO production. Also, FKA significantly reduced LPS-induced NO levels in a dose-dependent manner.

Figure 1. Effect of FKA on NO production in LPS-stimulated RAW 264.7 cells.

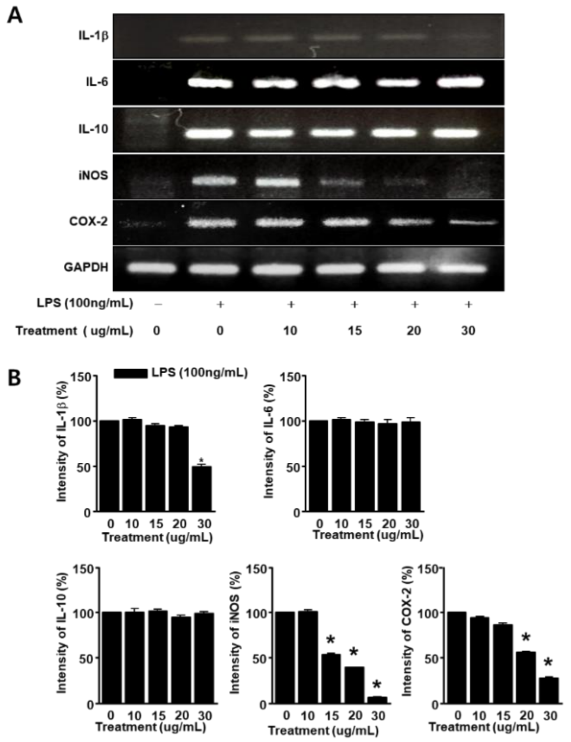


Note: (A)The cells were treated with 10-30 μg/mL FKA. Cell viability was determined via a cell viability assay. (B)The cells were treated with the presence or absence of 100 ng/mL LPS, and 10-30 μg/mL FKA. After 24 h, NO production was analyzed using the supernatant. Results are presented as means ± S.E of 3 independent experiments. *P<0.05 vs. the untreated group.

3.2. Anti-inflammatory effect of FKA on LPS-induced RAW 264.7 cells

To determine the effect of FKA on LPS-induced cytokines and inflammatory factors, we analyzed their mRNA expression, using RT-PCR. LPS-stimulated RAW 264.7 cells were treated with 10-30 μg/mL FKA for 24 h. As shown in Figure 2, treatment with 100 ng/mL LPS led to increased IL-1β, IL-6, IL-10, iNOS, and COX-2 expression, which was ameliorated by treatment with 30 μg/mL FKA. FKA treatment reduced IL-1β, iNOS, and COX-2 expression by 71, 95, and 80%, respectively.

Figure 2. Effect of FKA in LPS-induced cytokines and inflammatory factors in RAW 264.7 cells.

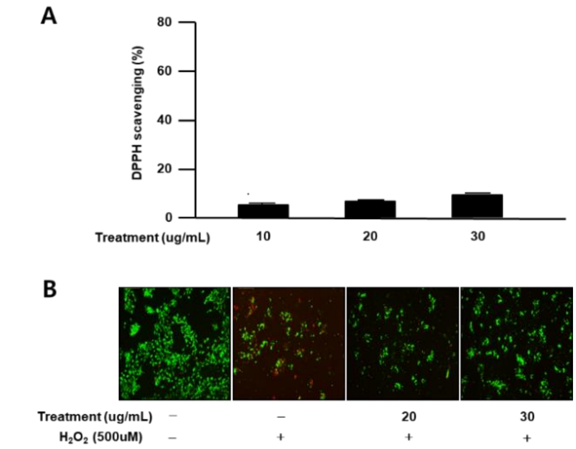


Note: (A)PCR products were loaded into agarose gel and stained with EtBr. The bands indicate expression levels of IL-1β, IL-6, IL-10, iNOS, and COX-2 mRNAs. (B)The graph shows the intensity of the bands relative to the LPS-treated group. * $P < 0.05$ vs. the LPS-only treatment. LPS, lipopolysaccharide; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.3. Effect of FKA on ROS generation

We assessed whether FKA can regulate DPPH free radical scavenging and exogenous ROS protection *in vitro*. As shown in Figure 3A, FKA slightly reduced DPPH free radicals. As shown in Figure 3B, H_2O_2-stimulated RAW 264.7 cells were treated with 20 and 30 μg/mL FKA for 6 h. H_2O_2 (500 uM) significantly reduced cell viability(red colors, death cells; green colors, live cells), whereas FKA significantly protected against exogenous ROS-induced cell death. Pretreatment with 20 and 30 μg/m FKA decreased H_2O_2 -induced cell death by 80 and 90%, respectively.

Figure 3. Effect of FKA in ROS generation.



Note: (A)The scavenging activity in DPPH radical. (B)The effects of FKA on cell viability were determined using the LIVE/DEAD cell viability assay kit. Morphological changes in exogenous ROS(H_2O_2)–simulated RAW 264.7 cells were observed using microscopy.

4. Discussion and Conclusion

The inflammatory response plays an important role in the body's defense mechanism, but an excess of it can be fatal. Anti-inflammatory agents are classified as steroids or non-steroids. Many steroids, including glucocorticoids, bind to glucocorticoid receptors to reduce swelling and inflammation. However, steroid administration to athletes is heavily regulated. Moreover, non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase(COX) enzymes to relieve pain, could be an alternative to athletes, but cause gastritis[9]. Therefore, alternative anti-inflammatory drugs for athletes are needed.

Reactive oxygen species(ROS) are cationic catalytic molecules inevitably generated during exercise, which simultaneously exert beneficial and deleterious effects on cells. Excessively elevated cellular ROS levels can lead to oxidative stress, that can lead to extensive cellular damages such as DNA breakdown, lipid peroxidation, and protein denaturation, which can be fatal to muscles[10].

In this study, we found that FKA treatment regulated NO production in LPS-stimulated RAW 264.7 cells, confirmed by decreased IL-1β, iNOS, and COX-2 expression. Moreover, FKA protected against exogenous ROS-induced cell death. These results indicate that FKA has anti-oxidant and anti-inflammatory effects, thus, can possibly contribute to the advancement of exercise capacity.

In conclusion, we have clearly demonstrated the anti-oxidant and anti-inflammatory effect of FKA in vitro. Our results suggest that FKA not only reduced iNOS, COX-2, and IL-1β expression, but protected against exogenous ROS-induced cell death. Therefore, FKA is a promising therapeutic agent for acute and chronic inflammation. However, the mechanism of its effect on exercise-induced inflammation requires further elucidating.

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