Application of stabilizer improves stability of nanosuspended branched-chain amino acids and anti-inflammatory effect in LPS-induced RAW 264.7 cells

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Abstract This study investigated the use of polyglyceryl esters (PGE) as stabilizer in improving stability and anti-inflammatory activity of nanosuspended branched-chain amino acids (BCAAs). BCAAs nanosuspended with stabilizer (BS) exhibited improved stability at concentration of 5% saturation level during storage as compared to BCAAs nanosuspended with aqueous solution (BA). Additionally, anti-inflammatory activity of BS was found to be greater than that of BA. Nitric oxide scavenging activity was found to be dose-dependent, with activity of BS in sodium nitroprusside system being significantly higher than that of BA ($p < 0.05$) at 2.5–20 mg/mL. BS also possesses greater inhibitory activity on production of pro-inflammatory factors including inducible nitric oxide synthase, cyclooxygenase-2 (COX-2), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells through suppressed phosphorylation of p65 subunit of NF- κ B at 0.5, 2, 8 mg/mL. These results suggest that PGE used as stabilizer improves solubility and biological activity of nanosuspended BCAAs.

Keywords High pressure homogenization · Stabilizer · Branched-chain amino acids · Anti-inflammatory · RAW 264.7 macrophages

Introduction

Branched-chain amino acids (leucine, isoleucine, and valine) are well known as the essential amino acids for humans. BCAAs have varying functions, such as metabolism in muscle cells, as precursors of other amino acids and proteins, and as part of the immune system [1]. However, as BCAAs have low water solubility, their application in food and pharmaceutical products is limited. Previous experiments attempted to make high concentration of BCAAs and to improve solubility of samples using high-pressure homogenization resulting in complex formation with stabilizer [2, 3].

High pressure homogenization technology is widely used in industries such as food, cosmetics, and pharmaceuticals. High-pressure homogenization improves characteristics such as quality, shelf-life, and bioavailability of active compounds [4]. Other research has reported that nanosuspended material manufactured through high-pressure homogenization exerted improved bioactivity such as anti-inflammatory and anti-oxidant activity [5–7]. Solubility of materials which have poor solubility in aqueous solution could be improved to saturation solubility in nanoemulsion or nanosuspension by application of high pressure homogenization. However, in the case of materials with poor solubility, crystal formation and aggregation could occur during storage. The use of stabilizers helps preserve the quality of food products against external conditions, maintain efficacy, improve stability, and exhibit a masking effect [8]. Polyglyceryl ester (PGE) is an

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effective stabilizer and is widely used as a water-in-oil emulsifier [9].

Inflammation is a biological immune response of the body that is triggered by various external stimuli such as bacterial infection, and tissue injury [10]. The inflammatory process involves immune cells and inflammatory mediators. Prolonged inflammation leads to chronic or autoimmune diseases that are caused by pro-inflammatory factors and pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF- α) [11, 12]. Macrophages are one of immune cells and play a crucial role in inflammatory responses. Macrophages are activated by stimuli such as lipopolysaccharides (LPS), and excessive production of pro-inflammatory factors such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandin e₂ (PGE₂), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines is induced. LPS-stimulated inflammation progression in macrophages is mainly mediated by nuclear transcription factor kappa-B (NF- κ B) activation. NF- κ B, which is composed of p65 and p50 subunits, controls the transcription of multiple genes, including inducible enzymes (iNOS and COX-2) and pro-inflammatory cytokines [13, 14]. Recently, many studies have been carried out on potential anti-inflammatory agents to regulate pro-inflammatory mediators that are excessively generated during inflammatory responses. Therefore, the objective of this study was to evaluate the use of PGE, as a stabilizer, in improvement of stability during storage and anti-inflammatory effect of BCAAs nanosuspended with aqueous solution.

Materials and methods

Materials

Phosphate-buffered saline (PBS), water, phenol-free Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Hyclone Laboratories, Inc (South Logan, UT, USA). BCAAs (leucine, iso-leucine, valine), and polyglyceryl esters were purchased from Ilshin Wells (Chungcheongbuk-do, Cheongju, Korea). Specific primers for PCR were purchased from Bionics (Seoul, Korea). RNeasy Mini Kit for total RNA isolation was purchased from Qiagen (Hilden, Germany). RIPA buffer, protease, phosphate inhibitor cocktail (EDTA-free), Modified Lowry Protein Assay Kit, RevertAidTM First Strand cDNA Synthesis Kit, Taq PCR Master Mix, SYBR green PCR Master Mix, and all other PCR reagents were purchased from Thermo Scientific Pierce (Waltham, MA, USA). Primary antibodies for iNOS, COX-2, NF- κ B p65, p-NF- κ B p65, and β -actin, and

secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Other reagents and devices for western blotting were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Nanosuspended BCAAs solutions were manufactured as described previously [2] with slight modifications. In detail, to prepare nanosuspended BCAAs with or without PGE in aqueous, a mixture of BCAAs (L-leucine:L-iso-leucine:L-valine = 2:1:1) with PGE (0.2%, w/w) or without PGE, was directly dissolved in water to be a final concentration of 4% (w/w) and stirred for 1 h at room temperature. Solutions were sonicated for 5 min at 25 °C. To prepare the samples, the solutions were preheated to 70 °C to prevent crystal formation and followed by homogenization over 5 cycles at 70 °C, 1000 bar using a thermostatic high-pressure homogenizer (MN400BF, Micronox, Seongnam, Korea). BCAAs nanosuspended with stabilizer (BS) and BCAAs aqueous nanosuspension (BA) were stored at room temperature until analysis. Concentrations of final samples were determined by high performance liquid chromatography (HPLC).

Evaluation of stability of BS and BA

Stability of solutions (BS and BA) was evaluated by measuring the concentration of soluble BCAAs at room temperature during storage period (0, 1, 5, 10, and 20 days). To determine the stability, each solution was maintained at a final concentration of 5% saturation level using a thermostatic high-pressure homogenizer as described previously. To measure concentration, samples obtained from the middle layer of the solution were filtered with syringe filter units (0.02 μ m) to remove crystallized BCAAs. After filtration, concentration of BCAAs was determined with HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, autosampler, Zorbax Eclipse-AAA column (5 mm, 150 $\times$ 4.6 mm, Agilent Technologies), column thermostat, and UV detector. Samples were automatically derivatized with o-phthalaldehyde (OPA) reagent by programmed autosampler, and analysis was carried out as described by manufacturer.

Cell culture conditions

The murine macrophage RAW 264.7 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cell line was maintained in phenol-red free DMEM medium containing 10% heat inactivated FBS, penicillin

(100 U/mL), and streptomycin (100 µg/mL). RAW 264.7 cells were kept in a CO₂ incubator (MCO-18AIC, Sanyo, Moriguchi, Osaka, Japan) containing 5% CO₂ at 37 °C and subcultured at 7–80% confluence.

Nitric oxide scavenging activity of BS and BA

Sodium nitroprusside (SNP) assay was performed to determine the NO scavenging activity of BS and BA [15]. Aliquot of 10 mM SNP (Sigma-Aldrich) dissolved in 0.1 M phosphate buffer was mixed with equal volume of different concentrations of BS and BA (0, 1.25, 2.5, 5, 10, and 20 mg/mL) and incubated in a 37 °C water bath for 18 h. The amounts of nitric oxide produced were determined with Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water). The supernatant of sample-treated SNP mixture reacted with equal volume of Griess reagent and incubated at room temperature in dark for 15 min. Absorbance of each well was measured at 540 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA), following which NO concentration was calculated according to concentration of sodium nitrite (NaNO₂) in water as a standard curve.

Inhibitory effect of BA and BS on NO production in LPS-induced RAW 264.7 cells

RAW 264.7 cells were plated at in 96-well plates (2×10^5 cells/well) and subsequently treated with 50 µL of different concentrations each of BS and BA (0, 0.625, 1.25, 2.5, 5, and 10 mg/mL) for 4 h, then treated with or without 50 µL of LPS (1 µg/mL) and incubated for 18 h at 37 °C in CO₂ incubator. NO concentration in culture medium was assessed using Griess reaction. Each aliquot of culture supernatant was reacted with equal volume of Griess reagent. After incubating at room temperature in dark for 15 min, the absorbance of each well was measured at 540 nm, using a microplate reader. Concentrations of NO were calculated according to concentration of sodium nitrite (NaNO₂) in phenol-free DMEM medium as a standard curve.

Cell viability of BA and BS in LPS-induced RAW 264.7 cells

Cytotoxicity of BA and BS on LPS-induced RAW 264.7 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays [16]. MTT assays were performed after measuring NO concentrations in medium. Aliquot of MTT solution (2.5 mg/mL in PBS) was added to the cells in 96-well plates and incubated at 37 °C for 4 h in CO₂ incubator. After incubation,

supernatants were removed and 200 µL of dimethyl sulfoxide (DMSO) was added to the wells to dissolve formazans. The absorbance of each well was measured at 570 nm using a microplate reader. Cytotoxic activities were calculated using the following equation:

$$\text{Cytotoxic activity (\%)} = [1 - (\text{absorbance in the sample} / \text{absorbance in the control})] \times 100$$

RT-PCR and real time quantitative PCR

RAW 264.7 cells were seeded (1×10^6 cells/well) in 6-well plate and subsequently different concentrations (0.5, 2, 8 mg/mL) of BS and BA groups were treated and incubated for 24 h. After incubation, cells were stimulated with or without LPS (1 µg/mL), respectively for 18 h. Total RNA was isolated using RNeasy Mini Kit and quantified using a microplate reader (Epoch, BioTek, Winooski, VT, USA). Equal amount of total RNA was synthesized into cDNA using commercial kit and synthesized cDNA was used as templates for RT-PCR and qPCR. The upstream and downstream primer sequences were as follows: iNOS, (sense 5'-CCCTTCCGAAGTTTCTGGCAGC-3' and antisense: 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3') COX-2, (sense 5'-CACTACATCTGACCCACTT-3' and antisense 5'-ATGCTCCTGCTTGAGTATGT-3) IL-1β, (sense 5'-CAGGATG-AGGACATGAGCACC-3 and antisense 5'-CTCTGCAGACTCAAACCTCCAC-3') IL-6, (sense 5'-AGTTGCCTTCTTGGA-3', antisense 5'-TTCTGCA-AGTGCATCATCGT-3') and β-actin (sense 5'-GTGGGCCGCCCTAGGCACCAG-3', antisense 5'-GGAGGAAGAGGATGCGGCAGT-3'). Final product of PCR mixture were amplified as follows: denaturation at 95 °C for 5 min and 95 °C for 30 s, annealing at specific temperatures for primers for 30 s, extension at 72 °C for 45 s (20–25 cycles) and final extension 72 °C for 10 min. The amplified cDNA were separated by 1.2% agarose gel electrophoresis and stained with DNA safestain (Thermo Scientific Pierce, Waltham, MA, USA). Separated bands in gel were visualized using UV visualizing table (Vilber Lourmat, Torcy, France). Real-time PCR assay was performed with SYBR green reagent. SYBR Green PCR mixture was amplified as follows: 95 °C for 2 min as initiation, and followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, with a final extension at 60 °C for 30 s. Amplified results were analyzed by delta–delta C_q (ΔΔC_q) method, and the purity of PCR products was evaluated with the melting curve from PCR reaction. β-Actin was used for normalization as internal control.

Western blot analysis

RAW 264.7 cells, pretreated with samples, were washed twice with cold PBS and lysed in cold RIPA buffer containing protease and phosphatase inhibitors. Cell lysates were centrifuged at $10,000\times g$ for 15 min at 4 °C (Micro 17TR, Hanil, Gimpo, Korea). After the cell debris were discarded, total protein concentrations of the supernatants were measured using Modified Lowry Protein Assay Kit (Bio-rad). The samples (40 µg of protein) were mixed with sample buffer. The mixtures were boiled at 95 °C for 5 min and separated using 10% polyacrylamide gel, then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk solution in tris-buffered saline Tween-20 (TBST) buffer for 1 h at room temperature, then probed overnight at 4 °C with diluted primary antibody of iNOS, COX-2, β -actin, p65, and p-p65 (1:2000) in 5% skim milk. After washing 5 times with TBST buffer, the membranes were subsequently probed with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase for 2 h at room temperature. After washing 5 times with TBST buffer, the blots were developed with enhanced chemiluminescence (ECL; Thermo Scientific Pierce) reagent.

Statistical analysis

All values are presented as mean and standard deviation (SD) of triplicates. Statistical analyses were performed using SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA). Student's *t* test was used to determine the differences between the groups, and One-way analysis of variance (ANOVA) was performed to analyze differences in multiple groups by Duncan post hoc test with *p* value < 0.05 considered statistically significant.

Results and discussion

Solubility of nanosuspended BCAAs during storage

Influence of PGE on solubility of BS and BA was investigated during storage period. As shown in Table 1, neither BS nor BA maintained initial solubility during storage period. Concentration of BA decreased significantly during storage period and solubility after 20 days was reduced to approximately 78.02% of its initial solubility. In the case of BS, concentration showed a slight decrease, with a solubility after 20 days of approximately 90.76% of its initial solubility. Furthermore, solubility of BS was significantly higher than that of BA on the same storage day. As reported previously [2], use of surfactants as stabilizers in nanosuspended BCAAs improves solubility and stability

Table 1 Changes of concentration of BCAAs during storage period

Storage period (day)	Concentration (%)	
	BS	BA
0	$^{z}4.98 \pm 0.01^a$	$^{z}4.96 \pm 0.02^a$
1	$^{z}4.94 \pm 0.01^{ab}$	$^{y}4.48 \pm 0.07^b$
5	$^{z}4.86 \pm 0.04^b$	$^{y}4.21 \pm 0.02^c$
10	$^{z}4.59 \pm 0.06^c$	$^{y}4.01 \pm 0.03^d$
20	$^{z}4.52 \pm 0.04^c$	$^{y}3.87 \pm 0.06^e$

Data expressed as mean $\pm$ S.D. of triplicate individual experiments
BS nanosuspended BCAAs with stabilizer, BA nanosuspended BCAAs

The values with different superscripts (z and y) indicates significant difference in same row (*p* < 0.05)

The values with different superscripts (a, b, and c) indicates significant difference in same column (*p* < 0.05)

during storage period as compared to established BCAAs nanosuspended with aqueous solution. These results suggest that highly concentrated BCAAs nanosuspended with aqueous solution could be prepared by thermostatic high-pressure homogenization. Further, although PGE as a stabilizer had no effect on initial state, PGE improves solubility during long-term storage and contributes to stability of highly concentrated nanosuspended BCAAs solution.

Nitric oxide scavenging activity of BS and BA in SNP system

The level of NO production was measured by SNP system in order to evaluate nitric oxide scavenging activity of BS in comparison to that of BA in vitro. In previous study, each BCAA inhibited the levels of NO production by up to about 30% in SNP system [17]. As shown in Fig. 1, BA and BS groups (2.5–10 mg/mL, respectively) showed significant scavenging activity in the amounts of generated NO in a dose-dependent manner (*p* < 0.05) and the BS group exhibited higher NO scavenging effects than the BA group. PGE used as stabilizer is known as a non-ionic surfactant, and there have been no previous studies on its functional ability. Chat et al. [18] have shown that complexes with surfactants exhibited enhanced ability, low solubility, and DPPH radical scavenging activity. Thus, these results suggest that BCAA nanosuspension containing PGE had enhanced NO scavenging activity.

Inhibitory effect of nitric oxide production in LPS-stimulated RAW 264.7 cells

Before determining the anti-inflammatory effect of both BS and BA ex vivo, the cell viability of BS and BA was measured by MTT assay. As shown in Fig. 2(A), cell

Fig. 1 Nitric oxide scavenging effect of BA and BS in SNP system. All values are represented mean $\pm$ S.D. of triplicate experiments. Bar labeled different letters represent significant differences. Significances were evaluated based on a one-way ANOVA after Duncan's multiple test ($p < 0.05$). Black bar, BCAA aqueous nano-suspension (BA); White bar, BCAA nano-suspended with stabilizer (BS); D.W, distilled water

viability was found to be dose-dependent (0–10 mg/mL), and was above 90% in all cases, indicating that both BA and BS showed no cytotoxicity against RAW 264.7 cells. Thus, the results of cytotoxicity of samples were excluded in subsequent experiments related to anti-inflammatory activity. To investigate inhibitory effect of BA and BS on NO production in LPS-induced RAW 264.7 cells, the levels of NO accumulation in culture media were determined by Griess reaction. As shown in Fig. 2(B), the levels of NO production significantly decreased in a dose-dependent manner in LPS-activated RAW 264.7 cells ($p < 0.05$). At 5 and 10 mg/mL concentrations, inhibitory effect on nitric oxide production of BS group was

significantly more enhanced than that of BA group ($p < 0.05$). In normal conditions, NO is a biological factor with the ability to regulate blood vessels and is associated with neurotransmitters related to cardiovascular and nervous systems. Further, it has been known that NO plays an important role in inflammatory response [19]. Immune-activated macrophages secrete appreciable levels of NO at inflammatory sites to reduce the inflammation. However, the overproduction of NO leads to critical diseases involved in various inflammation disorders [20]. Thus, the inhibition of NO production in LPS-stimulated RAW 264.7 cells is relevant to anti-inflammatory activity. According to Calder [21], BCAAs are essential for immune cells, related

Fig. 2 Effect of BA and BS on nitric oxide production in LPS-stimulated RAW 264.7 cells. (A) Cell viability of BA and BS, (B) Inhibitory effect of BA and BS on NO production; All values are represented mean $\pm$ S.D. of triplicate experiments. Bar labeled different letters represent significant differences. Significances were

evaluated based on a one-way ANOVA after Duncan's multiple test ($p < 0.05$). Dark gray bar, LPS positive, LPS negative; Black bar, BCAA aqueous nano-suspension (BA); White bar, BCAA nano-suspended with stabilizer (BS)

to protein synthesis, lymphocyte responsiveness, and support of other immune cell functions. As shown in the results, BCAAs exert inhibition of nitric oxide production and BS shows improved inhibitory activity of nitric oxide production in LPS-induced RAW 264.7 cells.

Effect of BS and BA on iNOS, COX-2 protein and mRNA expression

To investigate whether BS and BA groups affect iNOS, and COX-2 enzymes linked to pro-inflammatory mediators, the protein and mRNA levels of iNOS and COX-2 were examined by western blot analysis, RT-PCR, and real time qPCR. As shown in Fig. 3(B), mRNA expression levels of iNOS were significantly decreased at concentrations of ≥ 2 mg/mL, and BS showed greater inhibitory effect than BA ($p < 0.05$). Regarding protein levels of iNOS (Fig. 3), suppressive activity of BS was higher when compared to that of BA, and iNOS protein expression, particularly, was almost blocked at 8 mg/mL concentration of BS. De Simone et al. [22] reported that iNOS mRNA expression level was decreased in microglial cells, which were incubated with culture medium containing BCAAs under LPS-activated conditions. mRNA and protein expression of

COX-2 was significantly inhibited in a dose-dependent manner in BS groups as compared to that in BA groups, whereas only LPS-induced RAW 264.7 cells pretreated with 8 mg/mL sample of BS showed inhibitory effect on expression of COX-2 protein [Fig. 3(C)] ($p < 0.05$). Stimulated macrophages in inflammation induced expression of COX-2, an inducible pro-inflammatory enzyme involved in immune disorders [11]. Overexpression of COX-2 enzyme excessively produced prostaglandin, involved in pathogenesis of various inflammatory diseases, cancer cell invasion, and angiogenesis [23]. Many studies examined that anti-inflammatory agents have potential in investigation of inhibitory effect of COX-2 expression [7, 11]. The experimental results showed that BCAA nanosuspension decreased the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells and BS exerted greater suppressive effect compared to BA.

Effect of BS and BA on mRNA expression of pro-inflammatory cytokines

In the present study, LPS-activated RAW 264.7 cells were used to assess inhibitory effect of BS and BA, and mRNA expression levels of IL-1 β and IL-6 were determined by

Fig. 3 Effect of BA and BS on protein, mRNA expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages. (A) Protein levels of iNOS, (B) mRNA levels of iNOS, (C) Protein levels of COX-2, (D) mRNA levels of COX-2; Proteins were extracted from whole cell lysates. All values are represented mean $\pm$ S.D. of

triplicate experiments. Bar labeled different letters represent significant differences. Significances were evaluated based on a one-way ANOVA after Duncan's multiple test ($p < 0.05$); Black bar, BCAA aqueous nano-suspension (BA); White bar, BCAA nano-suspended with stabilizer (BS)

RT-PCR and real-time quantitative PCR. As shown in Fig. 4(A), mRNA expressions levels of pro-inflammatory cytokine IL-1 β was significantly decreased in LPS-induced RAW 264.7 cells pretreated with 0.5 and 2 mg/mL of BA as compared to that in LPS-positive groups. However, mRNA expression level in cells pretreated with higher concentration (8 mg/mL) of BA was significantly increased as compared to that in LPS-positive group. On the other hand, all concentrations of BS had inhibitory effect on IL-1 β mRNA expression levels in a dose-dependent manner. In case of cytokine IL-6 [Fig. 4(B)], mRNA expression levels in pretreated BS group also showed significant decrease in a dose-dependent manner, whereas cells pretreated with BA group had not shown inhibitory effect at higher concentrations. IL-1 β and IL-6 are key mediators as pro-inflammatory cytokines for host defense and autoimmune system [24]. Activated macrophages stimulated with LPS stimuli lead to overexpression of IL-1 β and IL-6, which are involved in up-regulation of inflammatory reaction [25]. When high levels of IL-1 β and IL-6 persist for a long time, it causes cell damage and immune malfunction, such as rheumatoid arthritis, inflammatory bowel disease, and Alzheimer's disease [26, 27]. Subramanian et al. reported that high pressure homogenization used in nanoemulsion system of aspirin increased water solubility, and anti-inflammatory activity by reducing expression of the pro-inflammatory cytokines (TNF- α , IL-1 α) in ear edema mouse model of induced inflammation [5].

Effect of BS and BA on phosphorylation of NF- κ B in LPS-induced RAW 264.7 cells

To identify suppressive effect of BS and BA related to transcriptional activity of NF- κ B in LPS-stimulated RAW 264.7 cells, phosphorylated protein expression of p65 subunit was measured by western blot analysis. As shown in Fig. 5, both BA and BS reduced protein expression of the phosphorylated form of p65 in a dose-dependent manner. NF- κ B activation can lead to multiple physiological processes such as cell apoptosis, cell proliferation, and inflammation [28]. Under inflammatory conditions, NF- κ B, which is made up of p65 and p50 subunits, regulates the transcription of multiple genes, such as various inducible enzymes (iNOS and COX-2) and pro-inflammatory cytokines [29]. In previous studies, anti-inflammatory agents were investigated by suppression of phosphorylated p65 involved in NF- κ B transcriptional activation [23, 30]. Hence, these results have shown that BCAAs exert potential anti-inflammatory activity by controlling phosphorylation of p65 subunit among NF- κ B transcriptional mechanism in LPS-induced RAW 264.7 cells, and BS has greater inhibitory effect compared to BA.

In conclusion, BCAAs nanosuspended with PGE using high-pressure homogenization showed stability at high concentration and enhanced anti-inflammatory activity in LPS-induced RAW 264.7 cells as compared to BCAAs aqueous nanosuspension. This was achieved by

Fig. 4 Effect of BA and BS on mRNA expression of IL-1 β and IL-6 cytokines in LPS-stimulated RAW 264.7 macrophages. (A) mRNA levels of IL-1 β , (B) mRNA levels of IL-6; All values are represented mean $\pm$ SD of triplicate experiments. Bar labeled different letters

represent significant differences. Significances were evaluated based on a one-way ANOVA after Duncan's multiple test ($p < 0.05$); Black bar, BCAA aqueous nano-suspension (BA); White bar, BCAA nano-suspended with stabilizer (BS)

Fig. 5 Effect of BA and BS on phosphorylation of p65 subunits in RAW 264.7 macrophages. Protein were extracted from whole cell lysates. These data were repeated with triplicate experiments; BCAA aqueous nano-suspension (BA); BCAA nano-suspension with stabilizer (BS)

suppression of pro-inflammatory mediators (NO, iNOS, COX-2, IL-1 β , IL-6) through inhibition of phosphorylation of p65 subunit-mediated nuclear transcriptional factor NF- κ B. This study suggested that polyglyceryl esters have potential as stabilizers to not only improve stability and biological activity, but also to apply poorly soluble compounds to industrial usage in nanosuspension systems using high-pressure homogenization.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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