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Protective effects of aminoethyl-chitooligosaccharides against oxidative stress and inflammation in murine microglial BV-2 cells

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ABSTRACT

The aim of this study was to investigate the antioxidant and anti-inflammatory activities of aminoethyl-chitooligosaccharides (AE-COS) in murine microglial cells (BV-2). At the high concentration of 100 μg/ml, the inhibition of reactive oxygen species, DNA, protein and lipid oxidation were determined in BV-2 cells, at the rates of 77%, 80%, 85% and 85%, respectively. Furthermore, AE-COS was studied for its capabilities against lipopolysaccharide-induced inflammatory responses in BV-2 cells. It was found that AE-COS reduced the level of nitric oxide (NO) and prostaglandin E_2 production by diminishing the expression of inducible NO synthase and cyclooxygenase-2 without significant cytotoxicity. Moreover, the inhibitory activities of AE-COS on generation of tumor necrosis factor-alpha and interleukin-1beta were performed. Collectively, these results indicate that AE-COS possess potential antioxidant and anti-inflammatory activities in brain microglia, and could be a useful therapeutic agent for the treatment of neuroinflammatory diseases.

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1. Introduction

Neuroinflammation is thought to be mainly associated with the activity of glial cells in immune surveillance and host defense. Under pathological conditions, inflammation of the brain is closely involved in pathogenesis of several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, human immunodeficiency virus-associated dementia and multiple sclerosis (Dheen, Kaur, & Ling, 2007; Krause & Muller, 2010). Microglia are the principal immune effector cells in the central nervous system. Upon phagocytosis of invading bacteria, microglia are activated and produce pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, and prostaglandin E_2 (PGE_2), as well as nitric oxide (NO) and reactive oxygen species (ROS), which are thought to contribute to neuronal injuries and progression of the neurodegenerative diseases (Block, Zecca, & Hong, 2007). Therefore, the modulation of microglial activation is an effective therapeutic approach against neurodegenerative diseases.

Chito-oligomers (COS) have attracted increasing attention due to absence of toxicity, and superior biocompatibility (Muzzarelli, Stanic, & Ramos, 1999). Aminoethyl-COS (AE-COS) have important biological properties in medicinal and pharmaceutical applications such as angiotensin converting enzyme inhibitory (Ngo, Qian, Je, Kim, & Kim, 2008), acetylcholinesterase inhibitory (Yoon, Ngo, & Kim, 2009) and anti-proliferative effects (Karagozlu, Kim, Karadeniz, Kong, & Kim, 2010). However, the antioxidant and anti-inflammatory activities of AE-COS remains to be evaluated. In this study, we examine the antioxidant and anti-inflammatory activities of AE-COS on murine microglial cells (BV-2 cells) under lipopolysaccharide stimulation.

2. Materials and methods

2.1. Materials

BV-2 cells were a gift from Professor Il-Whan Choi (Inje University, Gyungnam, Korea). Enzyme immunoassay reagents for cytokine assays were provided from R&D Systems (Minneapolis, MN, USA). Oligo (dT)15 primer, M-MLV reverse transcriptase, and GoTaq DNA polymerase were purchased from Promega (Madison, WI, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6, dimethyl sulfoxide (DMSO), [3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), $FeSO_4$, H_2O_2, ethylenediaminetetraacetic acid (EDTA), 2,4-dinitrophenyl hydrazine, diphenyl-1-pyrenylphosphine (DPPP), guanidine hydrochloride,

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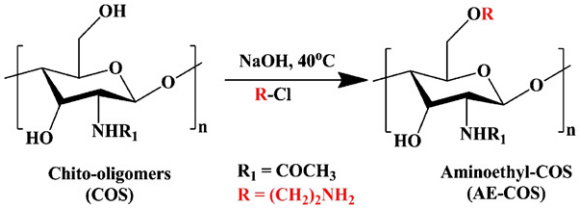

Scheme 1. Synthesis of aminoethyl-chito-oligomers (AE-COS).

agarose, fetal bovine serum (FBS) and Griess reagent were obtained from Sigma (St. Louis, MO, USA). Specific antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Amersham Pharmacia Biosciences (Piscataway, NJ, USA). All other reagents were of the highest grade available commercially. AE-COS was kindly donated by Doctor Dai-Nghiep Ngo (University of Science, Ho Chi Minh City, Viet Nam). AE-COS was produced as described by Ngo et al. (2008) (Scheme 1). AE-COS (molecular weight 800.79–4765 Da) was prepared from COS (800–3000 Da, deacetylation degree 90%) by grafting aminoethyl groups at C-6 position of pyranose ring.

2.2. Cell cytotoxicity, cellular ROS, DNA, proteins and lipids oxidation determination

BV-2 cells were cultured and maintained in DMEM media containing 5% (v/v) FBS, 100 μg/ml penicillin–streptomycin and 5% CO_2 at 37 °C. Cytotoxicity levels of samples on cells were measured using the MTT method as described by Vo, Kong, and Kim (2011) with slight modifications.

Intracellular formation of ROS was assessed according to a method described previously by employing oxidation sensitive dye DCFH-DA as the substrate (Qian, Jung, Byun, & Kim, 2008).

BV-2 cells were analyzed for the generation of lipid peroxidation products by modification of the DPPP method (Takahashi, Shibata, & Niki, 2001).

The degree of oxidation of the cell membrane proteins was assessed by determining the content of protein by carbonyl groups (Rajapakse, Kim, Mendis, & Kim, 2007).

Genomic DNA was extracted from BV-2 cells using AccuPrep® genomic DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer's protocol. Hydrogen peroxide mediated DNA oxidation was determined according to Ngo et al. (2011).

2.3. NO production assay

NO level in the culture supernatant was measured by the Griess reaction as described earlier (Cho, Lee, Kim, Ahn, & Je, 2011). In brief, BV-2 cells were plated at 1×10^5 cells/ml in 24-well culture plates, and stimulated for 24 h with LPS (1 μg/ml, final concentration) in the presence or absence of various concentrations of AE-COS. Aliquots (50 μl) of the supernatants were incubated with 50 μl of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% phosphoric acid] for 15 min. This was followed by measurement of absorbance values at 540 nm using a microplate reader (Tecan Austria GmbH, Grodig/Salzburg, Austria). The nitrite concentrations were calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

2.4. Enzyme-linked immunosorbent assay (ELISA)

For immunoassays, BV-2 cells were treated with different concentrations of AE-COS before stimulated with LPS (1 μg/ml, final concentration) for 24 h. The supernatant was collected, and production of PGE_2, IL-1β and TNF-α was quantified by sandwich immunoassays following the protocol of R&D systems.

2.5. Western blot analysis

Western blotting was used for detection of protein expression. Cells were washed three times with PBS and lysed in RIPA lysis buffer (NP-40, Sigma–Aldrich, USA). Equal amounts of protein were separated on 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and then blocked in TBS-T buffer (20 mM Tris, pH 7.6, 0.1% Tween 20) containing 5% (w/v) bovine serum albumin. After incubation with the appropriate primary antibody, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Following three washes in Tris-buffered saline-Tween (TBS-T), immunoreactive bands were visualized using the electrochemiluminescence (ECL) detection system. In a parallel experiment, nuclear protein was prepared using nuclear extraction reagents according to the manufacturer's protocol.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to analyze the mRNA expression of cytokine, iNOS, and COX-2. Total RNA was isolated using the TRIzol reagent (Invitrogen, Paisley, UK). Total RNA from the cells was reverse-transcribed using M-MLV reverse transcriptase to produce cDNA. RT-generated cDNAs encoding iNOS, COX-2, TNF-α, IL-1β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified by PCR using selective primers. Primer sequences used in this study were as follows: for iNOS, forward 5′-CCC-TTC-CGA-AGT-TTC-TGG-CAG-CAG-C-3′ and reverse 5′-GGC-TGT-CAG-AGC-CTC-GTG-GCT-TTG-G-3′; for COX-2, forward 5′-GGG-GTA-CCT-TCC-AGC-TGT-CAA-AAT-CTC-3′ and reverse 5′-GAA-GAT-CTC-GCC-AGG-TAC-TCA-CCT-G-3′; for TNF-α, forward 5′-ATG-AGC-ACA-GAA-AGC-ATG-ATC-3′ and reverse 5′-TAC-AGG-CTT-GTC-ACT-CGA-ATT-3′; for IL-1β, forward 5′-ATG-GCA-ACT-GTT-CCT-GAA-CTC-AAC-T-3′ and reverse 5′-TTT-CCT-TTC-TTA-GAT-ATG-GAC-AGG-AC-3′; for GAPDH, forward 5′-TGA-AGG-TCG-GTG-TGA-ACG-GAT-TTG-GC-3′ and reverse 5′-CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC-3′. Following amplification, portions of the PCR reactions were electrophoresed on an agarose gel.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of three independent determinations. The significance of differences between two samples was analyzed using the Student's *t*-test and *P*-value of <0.05 was considered as the level of statistical significance.

3. Results and discussion

3.1. Inhibition of free radical-mediated oxidation of cellular biomolecules by AE-COS

In this study, AE-COS was synthesized by grafting aminoethyl group into the hydroxyl group of COS at C-6 position of pyranose ring because the C-6 hydroxyl groups had the highest reactivity for aminoethylation. BV-2 cells were treated with various concentrations (10, 50 and 100 μg/ml) of COS and AE-COS for 24 h in order to assess the cytotoxic effect of COS and AE-COS by the MTT assay. The results showed that both COS and AE-COS exerted no significant toxic effect on BV-2 cells at all tested concentrations (Fig. 1(A)). Therefore, those concentrations of COS and AE-COS were used for all experiments.

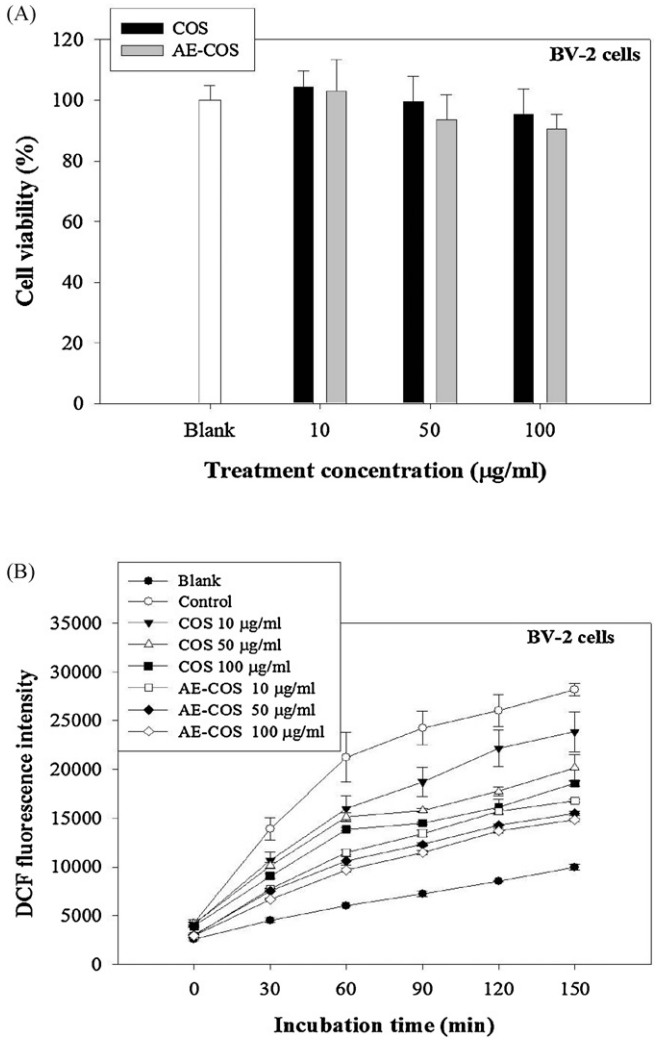

Fig. 1. Cell viability assessed by the MTT assay (A) and intracellular radical scavenging activity of COS and AE-COS in BV-2 cells (B). The results shown are representative of separate experiments performed in triplicate. Error bars represent the standard deviation (SD).

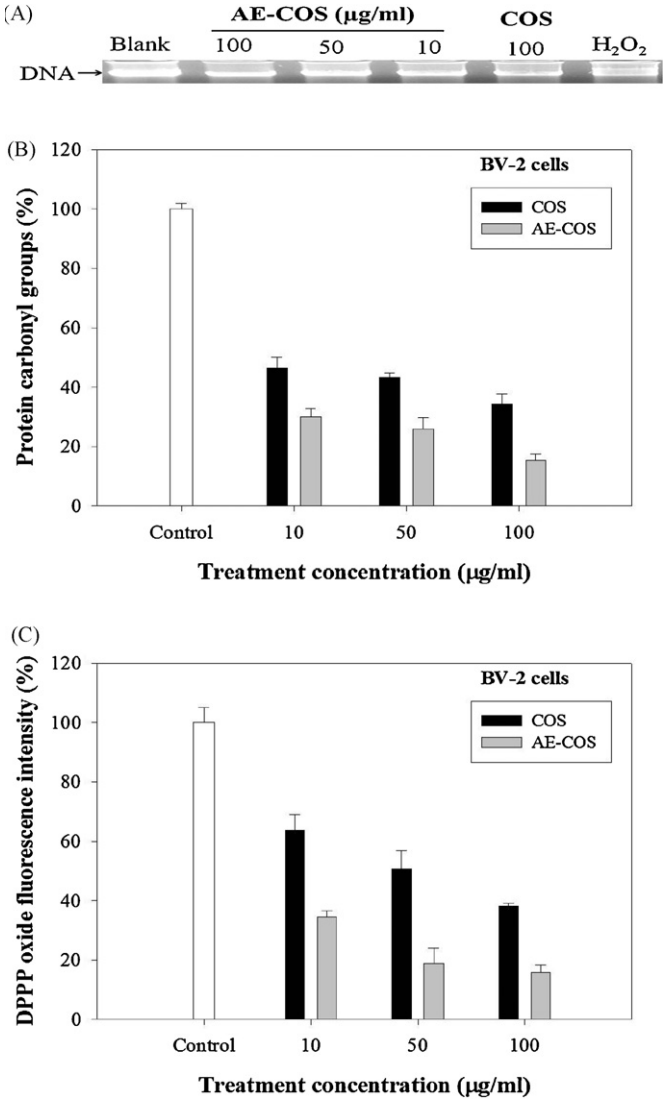

Fig. 2. DNA oxidative protection (A), protein oxidative protection (B) and lipid peroxidation protection (C) by COS and AE-COS in BV-2 cells. Results are means ± SD of three independent experiments.

BV-2 cells are commonly used to determine ROS-induced cellular issues as they are able to produce high amounts of ROS following stimulation (Arato et al., 2006). Intracellular ROS was measured using the fluorescence probe DCFH-DA. During labeling, non-fluorescent DCFH-DA dye that easily penetrates into the cells gets hydrolyzed by intracellular esterase to 2′,7′-dichlorodihydrofluorescein (DCFH), and DCFH traps inside the cells and gets oxidized by H_2O_2. As shown in Fig. 1(B), fluorescence emitted by 2′,7′-dichlorofluorescein (DCF) following ROS-mediated oxidation of DCFH was measured every 30 min up to 150 min incubation. Treatment with COS and AE-COS reduced the DCF fluorescence intensity, resulting the scavenging activity increased against intracellular ROS in a dose- and time-dependently. AE-COS was able to scavenge radicals significantly throughout the incubation time at all tested concentration. Especially, AE-COS reduced the DCF fluorescence intensity about 77% at 100 μg/ml after 30 min.

Reactive oxygen species are able to induce all forms of oxidative DNA damage (i.e. base-free sites, strand breaks, base modifications, and DNA-protein cross-link) (Mello, Hernandez, Marrazza, Mascini, & Kubot, 2006). In this research, DNA oxidation was carried out by combining effect of 200 μM Fe (II) and 2 mM H_2O_2 on the integrity of genomic DNA isolated from BV-2 cells. The inhibitory activity on DNA oxidative damage was subjected by DNA electrophoresis in the presence or absence of COS and AE-COS. After 10 min of reaction, almost all DNA was damaged in the control group treated only with Fe(II)–H_2O_2 (Fig. 2(A)). However, AE-COS exerted a protective effect on radical-mediated DNA damage in a clear dose-dependent manner. Furthermore, AE-COS inhibited more than 80% DNA damage at 100 μg/ml based on the intensity of DNA bands. In contrast, only about 25% DNA damage was inhibited by COS at 100 μg/ml.

Oxidation of cellular proteins by the Fenton reaction products generates the protein carbonyl groups such as aldehydes and ketones; those have been determined as oxidized protein markers and related to numerous diseases (Ngo, Lee, Kim, & Kim, 2009). The ROS-induced damage to proteins modifies amino acids such as proline, histidine, lysine and arginine producing carbonyl groups, which are used to measure protein oxidation. The inhibitors of cellular membrane proteins damage play a fundamental role in maintaining normal cell growth and biological functions. As shown in Fig. 2(B), the protein carbonyl groups formation was increased when BV-2 cells were exposed to hydroxyl radicals. When the cells in the reaction mixture were treated with varying concentrations of AE-COS and COS, the protein carbonyl groups formation was clearly decreased compared to *OH alone-treated control group. Through the protein carbonyl groups content, the results show that AE-COS

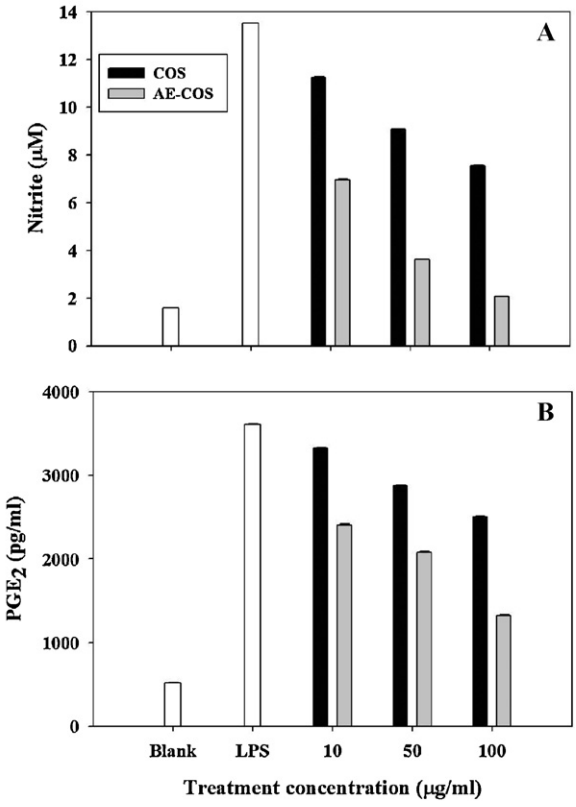

Fig. 3. Effects of COS and AE-COS on LPS-induced NO (A) and PGE_2 (B) production in BV-2 cells.

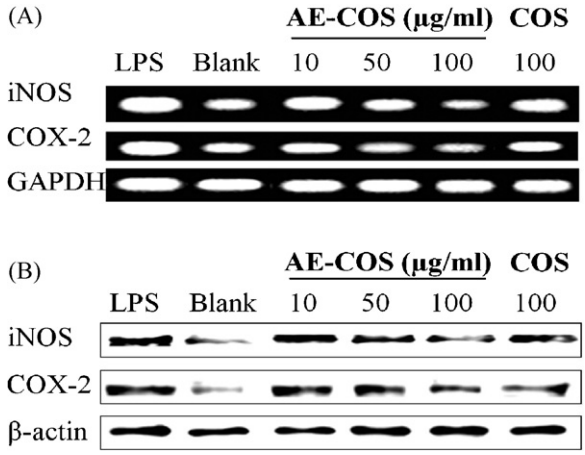

Fig. 4. Effects of AE-COS on LPS-induced mRNA and protein expressions of iNOS and COX-2 in BV-2 cells using RT-PCR (A) and Western blot (B) analysis, respectively.

inhibited about 85% of the formation of protein carbonyl groups in BV-2 cells at 100 μg/ml concentration. In addition, AE-COS inhibited oxidation of membrane protein that was significantly higher than that of COS at all the tested concentrations.

A sensitive fluorescence probe (DPPP) was used to measure the lipid hydroperoxide level of BV-2 cells produced by strong carbon-centered radical generating agent (AAPH). DPPP penetrated into the cell membranes and hydroperoxides (oxidation products of lipids) preferably react with DPPP to produce DPPP-oxide. DPPP itself is not fluorescent, but DPPP-oxide is fluorescent with high fluorescence (Takahashi et al., 2001). After 6 h of incubation with AAPH, the DPPP-oxide fluorescent intensity increased more than 1.5-fold due to carbon-centered radical-mediated membrane lipid peroxidation (Fig. 2(C)). The results show that an increase in DPPP-oxide fluorescence was inhibited by the treatment of AE-COS. At 100 μg/ml, AE-COS could inhibit membrane lipid peroxidation around 85%. Accordingly, AE-COS could inhibit membrane lipid peroxidation dose-dependently. In contrast, a comparatively low reduction in fluorescent intensity was observed in the presence of COS about 62% at 100 μg/ml.

3.2. Effect of AE-COS on production of NO, PGE_2 and expressions of iNOS and COX-2

The effects of AE-COS on NO and PGE_2 production in LPS-stimulated BV-2 cells via the Griess reaction and ELISA assay were elucidated, respectively. Obviously, LPS treatment increased the concentration of NO and PGE_2 in culture medium. Meanwhile, AE-COS significantly inhibited LPS-induced NO production in a concentration-dependent manner (Fig. 3(A)). Furthermore, the result of the PGE_2 assay demonstrated that AE-COS favorably reduced LPS-induced PGE_2 production at concentration of 100 μg/ml (Fig. 3(B)). To confirm whether the inhibition of NO and PGE_2 production is due to a decreased level of iNOS and COX-2, the effect of AE-COS on the expression of iNOS and COX-2 was determined. Fig. 4(A) shows the mRNA expression of iNOS and COX-2 in BV-2 cells by RT-PCR analysis. In this sense, the expression of iNOS and COX-2 mRNA was markedly increased after 24 h of LPS treatment compared to the blank without LPS and AE-COS. However, AE-COS decreased iNOS and COX-2 mRNA expression in LPS-stimulated BV-2 cells. In addition, the expression level of iNOS and COX-2 protein was also evaluated using Western blot analysis. Likewise, LPS-induced expression of iNOS and COX-2 protein was profoundly suppressed by AE-COS (Fig. 4(B)).

3.3. Effect of AE-COS on pro-inflammatory cytokines production and expression

To investigate the effects of AE-COS on the expression and production of pro-inflammatory cytokines, BV-2 cells were treated with LPS only or LPS with different concentrations of AE-COS. In the same way, the secretion of pro-inflammatory cytokines such as TNF-α and IL-1β was dramatically increased when incubation of BV-2 cells with LPS alone for 24 h compared to the blank group. However, the LPS-induced BV-2 cells dose-dependently produced lower levels of pro-inflammatory cytokines such as TNF-α, IL-1β after they were treated with AE-COS (10, 50 and 100 μg/ml) (Fig. 5(A) and (B)). These results indicated that AE-COS has the potential to inhibit LPS-induced production of TNF-α and IL-1β in BV-2 cells.

Neuroinflammatory response is also characterized via the production of cytokines such as TNF-α, IL-1β and IL-6. Several studies have also reported a key role for pro-inflammatory cytokine overproduction as a potential driving force for pathology progression in acute neurodegenerative disorders (Mrak & Griffin, 2005; Van Eldik, Thompson, Ralay Ranaivo, Behanna, & Martin Watterson, 2007). Additionally, the previous studies have evidenced that pro-inflammatory cytokines can induce iNOS/COX-2 expression, and subsequently induce large amounts of NO/PGE_2 production in activated neuroglia (Krady et al., 2005; Marcus, Karackattu, Fleegal, & Sumners, 2003). Consequently, the inhibition of cytokine production or function is a significant mechanism in alleviation of neuronal injury or death in neurodegenerative diseases. Here, we have found that AE-COS induced a dose-dependent suppression on the production and mRNA expression of TNF-α and IL-1β in LPS-stimulated BV-2 cells. This result indicates that the inhibitory activity of AE-COS against cytokine generation may lead to the alleviation of iNOS and COX-2 expression. According to these observations, AE-COS

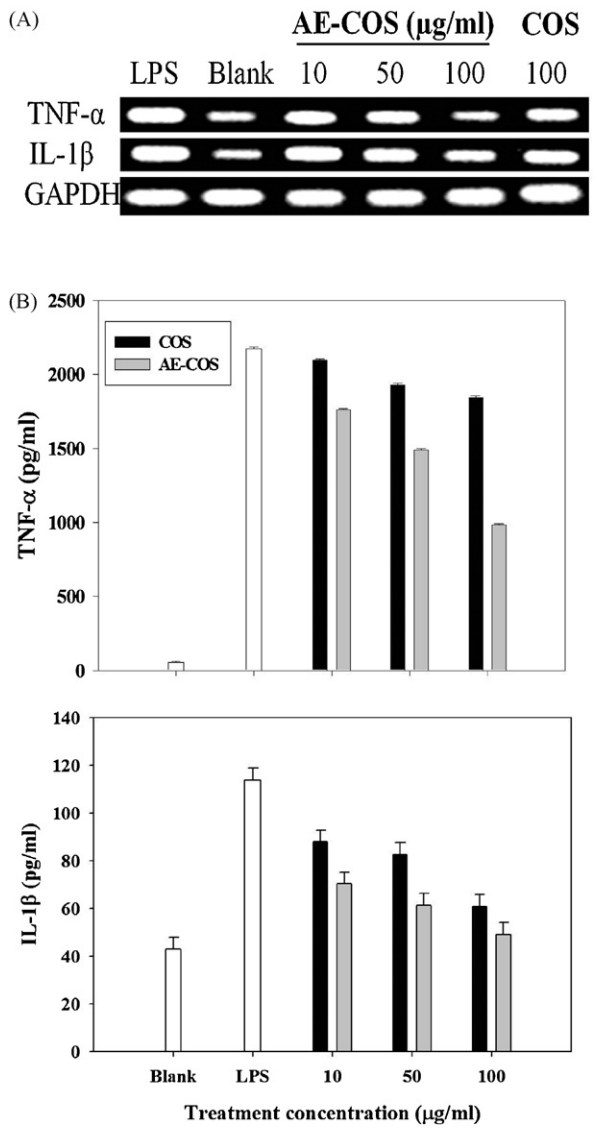

Fig. 5. Inhibitory effect of COS and AE-COS on the mRNA expression and production of inflammatory mediators TNF-α and IL-1β were determined by RT-PCR (A) and commercial ELISA kits (B) in BV-2 cells, respectively.

emerges as a promising therapeutic candidate to inhibit the LPS-induced microglial activation in the central nervous system.

4. Conclusion

According to the findings in this research, AE-COS exhibited antioxidant and anti-inflammatory activities in BV-2 murine microglial cells. AE-COS prevented oxidative damage to DNA, lipids, and proteins in BV-2 cells by free radicals. Moreover, AE-COS attenuated the productions of NO and PGE_2 by inhibiting iNOS and COX-2 expressions, respectively. The release and expression levels of pro-inflammatory cytokines, including TNF-α and IL-1β were also suppressed by AE-COS. They are confirmed to be deprived of toxicity, and might have therapeutic value for preventing neurodegenerative diseases.

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