Effects of *Armeniacae Semen* and Amygdalin on the Lipopolysaccharide-induced Prostaglandin E₂ Synthesis and Nitric Oxide Production in Mouse BV₂ Microglial Cells

Hyung-Jin Jung¹, Young-Sick Kim², Mal-Soon Shin², Chang-Ju Kim² and Youn-Sub Kim¹*

¹Department of Anatomy-Pointology, College of Oriental Medicine, Kyungwon University, Sungnam 461-701, ²Department of Physiology, College of Medicine, Kyunghee University, Seoul 130-701, Korea

**ABSTRACT**

*Armeniacae semen* has been used in traditional medicine for the treatment of pain and inflammatory diseases. Amygdalin is the major compound of *Armeniacae semen*, and it is used for treatment of pain and cancers. In the present study, we compared the effects of aqueous extract of *Armeniacae semen* and a solution of amygdalin extracted from *Armeniacae semen* on the lipopolysaccharide (LPS)-stimulated cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) mRNA expressions in mouse BV₂ microglial cells. We also compared the effects of these compounds on the prostaglandin E₂ synthesis and the nitric oxide production in mouse BV₂ microglial cells. In the present results, *Armeniacae semen* and amygdalin suppressed prostaglandin E₂ synthesis and nitric oxide production by inhibiting the LPS-induced enhancement of COX-2 mRNA and iNOS mRNA expressions in mouse BV₂ cells. For the COX-1 expression, *Armeniacae semen* showed more potent suppression effect compared to the amygdalin. However, amygdalin more potently suppressed the LPS-induced COX-2 mRNA expression compared to aqueous extract of *Armeniacae semen*. In the case of iNOS mRNA expression, *Armeniacae semen* and amygdalin showed similar suppressing effects. For the LPS-induced PGE₂ synthesis, amygdalin showed more potent suppressing effect, meanwhile, *Armeniacae semen* and amygdalin showed similar suppressing effect on NO production. Based on the present results, amygdalin may exert anti-inflammatory and analgesic effect through mainly the inhibition of COX-2 pathway, in contrast *Armeniacae semen* may exert such effect though both the inhibition of COX-2 and iNOS pathways.

**Key words:** amygdalin, *Armeniacae semen*, cyclooxygenase, inducible nitric oxide synthase, prostaglandin E₂, nitric oxide

**INTRODUCTION**

Herbs are annual, biennial, or perennial seed-producing soft-stem plants that can exert medicinal effects. The use of herbal medicine as comple-
mentary and alternative therapy is increasing rapidly throughout in the world. Armeniacae semen is the seed of Prunus armeniaca L. var. ansu MAXIM, which has been classified into Rosaceae. Armeniacae semen is known to have many therapeutic effects such as relieving fever, stopping cough, and quenching thirst. Armeniacae semen has been used in traditional medicine for the treatments of asthma, bronchitis, emphysema, constipation, nausea, leprosy, and leucoderma (Park et al., 1999; Hwang et al., 2003). Recently, Armeniacae semen has been reported to have anti-inflammatory and analgesic effects (Chang et al., 2005).

Amygdalin is one of major compounds of Armeniacae semen and this compound is abundant in the seeds of the Prunus genus, almonds, apricots, and the other rosaceous plants. Amygdalin is also known as vitamin B17 and it has also been named as a laetrile (Fukuta et al., 2003). Amygdalin is known to effectively relief the pain of cancer patients (Ellison et al., 1978; Shim et al., 2000).

During inflammatory conditions, various chemical mediators are released from the resident and infiltrating cells during inflammatory condition, and they sensitize peripheral nociceptors by initiating a cascade of events that changes the ionic conductance of the nociceptive peripheral terminals (Ito et al., 2001). Prostaglandins (PGs) are derived from arachidonic acid by the cyclooxygenase (COX) pathway and they play key roles in inflammatory processes, the sensitization of nociceptors, and for inducing pain (Minghetti and Levi, 1998; Garavito and DeWitt, 1999). COX is the rate-limiting enzyme in the synthesis of prostaglandins. This enzyme exists in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The two COX isoforms are very similar in structure, but they differ in the amino acid sequences at their active site. COX-1 is constitutively expressed in most cells for housekeeping functions. COX-2 is present at a low level under physiological conditions, and it is rapidly induced by various stimuli (Garavito and DeWitt, 1999). COX-2 is known to be responsible for the production of the high levels of PGs, and especially prostaglandin E2 (PGE2) in inflammatory conditions (Appleton et al., 1996).

Nitric oxide (NO) is endogenously generated from L-arginine by nitric oxide synthase (NOS), and NO plays an important role in the regulation of many pathophysiological processes (Brosnan et al., 1994). Several isoforms of NOS exist and these fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Both nNOS and eNOS are constitutively expressed, whereas iNOS is inducible in response to immunologic activation and its subsequent transcription (Bredt and Snyder, 1994). iNOS is responsible for the overproduction of NO in inflammatory conditions (Szabo, 1995).

In the present study, we compared the effects of an aqueous extract of Armeniacae semen and a solution of amygdalin that was extracted from Armeniacae semen on the lipopolysaccharide (LPS)-stimulated COX-1, COX-2, and iNOS mRNA expressions production in mouse BV2 microglial cells. We also compared the effects of these compounds on the prostaglandin E2 synthesis and the nitric oxide production in mouse BV2 microglial cells. For this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazoli um bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), prostaglandin E2 immunoassay, and NO detection were performed.

MATERIALS AND METHODS

Cell culture

The mouse BV2 microglial cells used in this experiment were cultured in Dulbeccos Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO2, 95% O2 in a humidified cell incubator.

Preparation of the aqueous extract of Armeniacae semen

The Armeniacae semen used in this experiment was obtained from the Kyungdong market (Seoul, Korea). After immersing it in 0.1% citric acid for 1 min, the Armeniacae semen was rinsed and dried at room temperature for 24 h. It was then pulverized with a crusher (Hanil, Seoul, Korea) and the fine powder was sifted from the course particles by using a mesh screen with a pore diameter of 2 mm. In order to obtain the aqueous extract of Armeniacae semen, the fine powder was sub-
subsequently heat-extracted with distilled water, pressure-filtered, and concentrated with a rotary evaporator (Eyela, Tokyo, Japan). The resulting 34.48 grams of powder (a yield rate of 6.88%) was obtained from 500 g of Armeniacae semen through lyophilization via a drying machine (Ilsin, Kyungkido, Korea) for 24 h.

**Extraction of amygdalin from Armeniacae semen**

Both 500 g of Armeniacae semen released from the shell and 10 L of 4% citric acid solution were refluxed for 2 h. After filtering this when it was still hot, the filtrate was passed through a column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. 4.2 g of amygdalin (a yield rate of 0.84%) was obtained by recrystallizing the extract with ethanol. The amygdalin was used after it had been determined to be over 99.0% pure by performing using high-pressure liquid chromatography (HPLC; Shiseido, Tokyo).

**MTT assay for cell viability**

Cell viability was determined by using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer’s protocol. In order to determine the cytotoxicity of an aqueous extract of Armeniacae semen and a solution of amygdalin, the cells were treated with the aqueous extract of Armeniacae semen or the solution of amygdalin at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, and 1,000 μg/ml for 24 h, respectively. The cultures of the control group were left untreated. 10 μl of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. 100 μl of solubilization solution was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and the absorbance at the test wavelength. The percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

**RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)**

RT-PCR was performed to identify the expressions of COX-1, COX-2, and iNOS mRNA. The total RNA was isolated from mouse BV2 microglial cells using RNAzol™ (TEL-TEST, Friendswood, TX, USA). 2 μl of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 10 min. 1 μl of AMV reverse transcriptase (Promega), 5 μl of 10 mM dNTP (Promega), 1 μl of RNasin (Promega), and 5 μl of 10×AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μl with using diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42°C for 2 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 1 μl, 4 μl of 10 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For the mouse COX-1, the primer sequences were 5′-AGTGCGGTCCAACCTTATCC-3′ (a 20-mer sense oligonucleotide) and 5′-CCGCAGGTGATACTGTCGTT-3′ (a 20-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5′-CCAGATGCTATCTTTGGGGAGAC-3′ (a 23-mer sense oligonucleotide) and 5′-CTTGCATTTGATGGTGGCTG-3′ (a 19-mer anti-sense oligonucleotide). For the mouse iNOS, the primer sequences were 5′-ATGAGGTACTCAGCGTGCTCCAC-3′ (a 23-mer sense oligonucleotide) and 5′-CCACAATAGTACAATACTTGG-3′ (a 24-mer anti-sense oligonucleotide). For the cyclophilin, which was used as the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTTCTTCGAC-3′ (a 20-mer sense oligonucleotide) and 5′-CCGCTATCAGGTGGCACTTGG-3′ (a 19-mer anti-sense oligonucleotide). For the cyclophilin, which was used as the internal control used in the study, the primer sequences were 5′-GAGTTACACCAGTCCAGCCAC-3′ (a 23-mer sense oligonucleotide) and 5′-CCACAATAGTACAATACTTGG-3′ (a 24-mer anti-sense oligonucleotide). For the cyclophilin, which was used as the internal control used in the study, the primer sequences were 5′-GAGTTACACCAGTCCAGCCAC-3′ (a 23-mer sense oligonucleotide) and 5′-CCACAATAGTACAATACTTGG-3′ (a 24-mer anti-sense oligonucleotide). For the cyclophilin, which was used as the internal control used in the study, the primer sequences were 5′-GAGTTACACCAGTCCAGCCAC-3′ (a 23-mer sense oligonucleotide) and 5′-CCACAATAGTACAATACTTGG-3′ (a 24-mer anti-sense oligonucleotide). The expected size of the PCR product was 381 bp for COX-1, 249 bp for COX-2, and 299 bp for cyclophilin.

For COX-1 and iNOS, the PCR procedure was carried out using a GeneAmp 9,600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: an initial denaturation step at 94°C for 5 min, followed by 35 amplification cycles with each one consisting of a denaturation step at 94°C for 30 sec, an annealing step at 58°C for 30 sec,
and an extension step at 72°C for 30 sec, with an additional extension step done at the end of the procedure at 72°C for 10 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were done. For COX-2, the PCR procedure was carried out under identical conditions except that 30 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically by using Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

**PGE2 assay**

The assessment of PGE2 synthesis was performed using a commercially available PGE2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). 100 μl of supernatant from each culture medium and the standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE2 antibody and peroxidase-conjugated PGE2 were then added to each well, and the plate was incubated with shaking at room temperature for 1 h. The wells were drained and washed, and 3,3,5,5-tetramethylbenzidine/hydrogen peroxide solution was then added to the wells. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H2SO4. The absorbance of the content of each well was then measured at 450 nm.

**Measurement of NO generation**

In order to determine the effect of an aqueous extract of *Armeniacae semen* and a solution of amygdalin on NO synthesis, the amount of nitrite (NO2−) in the cell-free culture supernatant was measured by employing a commercially available NO detection kit (Intron, Inc., Seoul, Korea). After collecting 100 μl of supernatant, 50 μl N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. 50 μl N2 buffer was then added and the plate was incubated at room temperature for 10 min. The absorbance of the contents of each well was measured at 540 nm. The nitrite concentration was calculated from a standard nitrite curve.

**Statistical analyses**

The results are expressed as the mean±standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncans post-hoc test using SPSS (version 11.5). Differences were considered statistically significant at p<0.05.

**RESULTS**

**Effect of aqueous extract of Armeniacae semen and amygdalin on cell viability**

The viability of cells incubated with aqueous extract of *Armeniacae semen* at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, and 10,000 μg/ml for 24 h were 102.84±1.81%, 102.97±0.86%, 103.96±1.45%, 90.05±0.97% (p<0.05), and 79.81±0.47% (p<0.05) of the control value, respectively. The MTT assay revealed that aqueous extract of *Armeniacae semen* exerted no significant cytotoxicity until it was at a concentration of 1 mg/ml in mouse BV2 microglial cells.

The viability of cells incubated with amygdalin at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, and 10,000 μg/ml for 24 h were 110.70±1.53%, 105.61±1.74%, 100.00±4.61%, 99.64±1.19%, and 85.54±1.16% (p<0.05) of the control value, respectively. The MTT assay revealed that amygdalin exerted no significant cytotoxicity until it was at a 10 mg/ml in mouse BV2 microglial cells. *Armeniacae semen* showed 10 fold potent cytotoxicity compared to amygdalin (Fig. 1).

**Effect of aqueous extract of Armeniacae semen and amygdalin on the expressions of COX-1, COX-2, and iNOS mRNA**

RT-PCR analysis of the mRNA levels of COX-1, COX-2, and iNOS was performed. In the present study, the mRNA level of COX-1, COX-2, and iNOS in the control cells was set as 1.00.

The level of COX-1 mRNA following treatment with 2 μg/ml LPS for 6 h was 0.94±0.06. It was decreased to 0.48±0.02 (p<0.05), 0.72±0.08 (p<0.05), and 0.35±0.04 (p<0.05) in cells treated with 100 μg/ml aqueous extract of *Armeniacae semen*, 100 μg/ml amygdalin, and 10 μg/ml aspirin 1 h prior to the 2 μg/ml LPS exposure, respectively. LPS treatment exerted significant effect on the COX-1
Effects of aqueous extract of Armeniacae semen and solution of amygdalin on viability of mouse BV2 microglial cells. (A) Control, (B) 1 μg/ml, (C) 10 μg/ml, (D) 100 μg/ml, (E) 1,000 μg/ml, (F) 10,000 μg/ml. *represents p < 0.05 compared to the control.

MnRNA expression in mouse BV2 microglial cells. Pretreatment with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin significantly suppressed COX-1 mRNA expression. Armeniacae semen showed more potent suppressing effect on COX-1 mRNA expression compared to Amygdalin.

The level of iNOS mRNA was significantly increased to 5.53±0.53 (p < 0.05) following 2 μg/ml LPS treatment for 6 h, while it was decreased to 2.20±0.22 (p < 0.05), 3.34±0.49 (p < 0.05), and 2.80±0.39 (p < 0.05) in cells treated with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin 1 h prior to the 2 μg/ml LPS exposure, respectively. LPS treatment significantly enhanced iNOS mRNA expression in mouse BV2 microglial cells. Pretreatment with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin suppressed LPS-induced iNOS mRNA expression. Armeniacae semen and amygdalin showed similar suppressing effect on LPS-induced iNOS mRNA expression (Fig. 2).

Effect of aqueous extract of Armeniacae semen and amygdalin on PGE2 synthesis
From the results of the PGE2 immunoassay, after 24 h of exposure to 2 μg/ml LPS, the amount of PGE2 from the culture medium was increased from 35.33±1.20 pg/ml to 53.67±9.06 pg/ml (p < 0.05). It was decreased to 47.00±3.46 pg/ml (p < 0.05), 42.17±5.17 pg/ml (p < 0.05), and 34.5±0.76 (p < 0.05) by the treatment with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin, respectively. LPS treatment increased PGE2 synthesis in mouse BV2 microglial cells. Pretreatment with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin significantly suppressed LPS-induced PGE2 synthesis. Amygdalin showed more potent suppressing effect on LPS-induced PGE2 synthesis (Fig. 3).

Effect of aqueous extract of Armeniacae semen and amygdalin on NO production
From the results of the NO detection assay, after 24 h of exposure to 2 μg/ml LPS, the amount of nitric oxide (NO) significantly enhanced COX-2 mRNA expression in mouse BV2 microglial cells. Pretreatment with 100 μg/ml aqueous extract of Armeniacae semen, 100 μg/ml amygdalin, and 50 μg/ml aspirin suppressed LPS-induced COX-2 mRNA expression. Amygdalin showed more potent suppression effect on LPS-induced COX-2 mRNA expression compared to Armeniacae semen.

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Effect of aqueous extract of Armeniacae semen and amygdalin on NO production
From the results of the NO detection assay, after 24 h of exposure to 2 μg/ml LPS, the amount of ni-
trite was increased from 6.63±0.20 μM to 45.97±
4.30 μM (p<0.05). It was decreased to 33.20±1.36
μM (p<0.05), 35.19±1.68 μM (p<0.05), 30.22±
2.12 μM (p<0.05) by treatment with 100 μg/ml
aqueous extract of Armeniacae semen, 100 μg/ml
amygdalin, and 50 μg/ml aspirin, respectively. LPS
treatment increased NO production in mouse BV 2
microglial cells. Pretreatment with 100 μg/ml aque-
ocous extract of Armeniacae semen, 100 μg/ml amyg-
dalin, and 50 μg/ml aspirin significantly suppressed
LPS-induced NO production. Armeniacae semen
and amygdalin showed similar suppressing effect
on LPS-induced NO production (Fig. 4).

**DISCUSSION**

Amygdalin is known as an important constituent
of Armeniacae semen. The aqueous extract of
Armeniacae semen contains about 10% of amygdalin. Armeniacae semen was exerted 10 fold potent cytotoxicity compared to amygdalin, in this study.

LPS is derived from the cell walls of gram-negative bacteria and mediates many of the inflammatory sequelae of infection (Frost et al., 2002). LPS initiates a number of major cellular responses that play a vital role in the pathogenesis of inflammatory responses including the activation of inflammatory cells and the production of cytokines and other mediators. In the present study, microglial cells activated by LPS produced a large amount of PGE2 and NO, which are known as critical factors in determining the final outcome of a microglial reaction to pathological stimuli. PGs synthesis and NO production are markedly increased when the inducible isoform of COX and NOS are expressed.

COX-1 isozyme is a housekeeping protein in most tissues, and it catalyzes the synthesis of PGs for normal physiological functions. COX-1 is expressed at a constant level, and this level does not fluctuate in response to various stimuli. In contrast, an inducible isoform COX-2 expression is rapidly stimulated by tumor promoters, growth factors, cytokines, and pro-inflammatory molecules in variety of cell types (Minghetti and Levi, 1998). COX-2 is known to be responsible for the production of the high levels of PGs in several pathological conditions such as inflammation, and it is the major isofrom expressed in inflammatory cells including microglia (Appleton et al., 1996). Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, inhibit both isoforms of COX, and they exert their beneficial effects by inhibiting of COX-2 and deleterious effects inhibiting of COX-1. When we consider that NSAIDs constitute one of the largest groups of prescribed drugs worldwide, searching for selective inhibitors of COX-2 will provide extensive clinical benefits in the future (Mitchell et al., 1995).

The present results show that Armeniacae semen and amygdalin inhibited COX-1 mRNA expression in mouse BV2 microglial cells. Armeniacae semen inhibited COX-1 mRNA expression more potently than amygdalin. These results suggest that amygdalin has a relative low potency of side effects such as peptic ulcer formation and renal dysfunction associated with COX-1 inhibition compared to Armeniacae semen.

The present results show that the expression of COX-2 mRNA in mouse BV2 microglial cells was significantly increased by LPS treatment, and that aqueous extract of Armeniacae semen and amygdalin inhibited LPS-induced COX-2 mRNA expression. Amygdalin had the more potent inhibitory effect on LPS-induced expressions of COX-2 and production of PGE2 compared to the aqueous extract of Armeniacae semen in mouse BV2 microglial cells. Chang et al. (2005) suggested that anti-inflammatory and analgesic effects of Armeniacae semen through inhibition of COX-2 mRNA in mouse BV2 microglial cells may depend on amygdalin content.

PGE2 synthesis in mouse BV2 microglial cells was increased by LPS treatment and Armeniacae semen and amygdalin suppressed LPS-induced PGE2 synthesis. The suppressive effect on LPS-induced PGE2 synthesis was more potent in amygdalin compared to Armeniacae semen.

NO production through the iNOS pathway is increased in inflammatory diseases, and excessive NO production induces cellular injury. Expression of the iNOS gene is increased in several pathophysiological conditions, and it produces large amounts of NO in response to inflammatory signals from such molecules as the cytokines and LPS (Mitchell et al., 1995; Minghetti and Levi, 1998). The present re-
results showed that iNOS mRNA expression in BV2 microglial cells was increased by LPS, and that Armeniacae semen extracts and amygdalin inhibited the LPS-induced iNOS mRNA expression.

Armeniacae semen and amygdalin showed a similar inhibiting effect on LPS-induced iNOS mRNA expression in mouse BV2 microglial cells. NO production in mouse BV2 microglial cells was enhanced by LPS treatment, and Armeniacae semen and amygdalin suppressed LPS-induced NO production as similar efficacy.

Based on the present result, amygdalin may exert anti-inflammatory and analgesic effect though mainly the inhibition of COX-2 pathway, in contrast Armeniacae semen may exert anti-inflammatory and analgesic effect though both the inhibition of COX-2 and iNOS pathways. The present study suggests that amygdalin extracted from Armeniacae semen is much safer and reliable agent for the treatment and inflammatory diseases than aqueous extract of Armeniacae semen.

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