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Anti-osteoarthritis effects of Pomegranate, *Eucommiae* cortex and *Achyranthis radix* extracts on the primary cultured rat articular chondrocytes

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Abstract

Objectives : The objective of present study is to evaluate anti-arthritic effects of dried pomegranate concentrate powders (PCP), *Eucommiae Cortex* aqueous (EC) and ethanolic (ECe) extracts, *Achyranthis Radix* aqueous (AR) and ethanolic (ARe) extracts on the primary cultured rat articular chondrocytes.

Methods : MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide) assay was performed cytotoxic effect of test substances. In addition, anti-inflammatory effects were also observed on the lipopolysaccaride (LPS) treated chondrocytes through prostaglandin E_2 (PGE_2) production and 5-lipoxygenase (LPO) activities, and inhibitory effects on metalloproteinase (MMP)-2 and MMP-9 activities were observed on the recombinant human interleukin (rhIL)-1α treated chondrocytes with their extracellular matrix (ECM) related mRNA expressions - collagen type II, SOX9 and aggrecan.

Results : As results, ECe and ARe showed obvious cytotoxicity against primary cultured rat articular chondrocytes at a dose level of 10 mg/ml, respectively. However, no obvious cytotoxic effects of PCP, EC and AR were demonstrated at a dose level of 10 mg/ml, on the primary cultured rat articular chondrocytes. In addition, treatment of LPS 50 μg/ml induced significant increases of PGE_2 contents and 5-LPO activities indicating inflammatory responses of the primary cultured rat articular chondrocytes, and also decreases of cell viabilities, increases of MMP-2 and MMP-9 activities with decreases of extracellular matrix (ECM) related collagen type II, SOX9 and aggrecan mRNA expressions were observed by treatment of rhIL-1α 50 ng/ml, suggesting damages on the primary cultured rat articular chondrocytes and related ECM degradations. However, these inflammatory responses and related ECM degradations were inhibited by pretreatment of all test substances, in order of PCP > ECe > ARe > EC > AR, and rhIL-1α induced chondrocytes deaths are inhibited by treatment in order of PCP > EC > AR > ECe > ARe.

Conclusions : Taken together, it is expected that mixed formulation of PCP as main components with

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appropriate proportion of EC and AR as additional components will be achieved a potent alternative medicinal food for osteoarthritis.

Key words : Primary cultured rat articular chondrocytes, Osteoarthritis, Dried pomegranate concentrate powders, *Eucommiae Cortex*, *Achyranthis Radix*, cytotoxicity

I. Introduction

Osteoarthritis (OA) is characterized by loss of articular cartilage components, mainly proteoglycans (PGs), leading to tissue destruction and hypocellularity, eventually resulting in loss of joint function[1]. In particular, the proinflammatory cytokines have been shown to stimulate cartilage resorption and inhibit the synthesis of new matrix components in articular cartilage tissue[2,3]. In addition, cytokines cause increases in prostaglandin E_2 (PGE_2) and nitric oxide release from chondrocytes[4,5], and reports have implicated both PGE_2 and nitric oxide involvement in the process of cartilage matrix turnover[6,7]. Consequently, there is a pressing need to develop disease-modifying OA drugs[8], and currently, the primary approach in the clinical treatment of OA involves the use of nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics and hyaluronan, which allow symptomatic relief, but provided no apparent disease-modifying effect[9]. In some instances, NSAIDs may even be deleterious effects; they inhibited the synthesis of PG, which play a crucial role in maintaining the function of the cartilage[9]. Therefore, there is a critical need to develop alternative agents that can prevent the destruction of cartilages or stimulate its proper repair. In these aspects, various efforts have been trialed that search for effective cartilage preserve agents through natural sources or mixed formulations with medicinal foods to achieve more potent anti-arthritic agents[10-12].

Pomegranate is a rich source of crude fibers, pectin, sugars, and several tannins[13]. In addition, it has been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract[14,15]. Furthermore, the powerful and favorable chondrocytes protective effects of pomegranate through their potent antioxidant and anti-inflammatory effects have been warranted, recently[16-19]. *Eucommiae Cortex* is a dried stem bark of *Eucommia Ulmoides* Oliver (*Eucommiaceae*), and has been used as a top-grade medicine in Korean medicine for antihypertensive, anti-inflammatory, antiviral, invigorating the kidney and hepatoprotective activities without side effects[20-22]. Particularly, anti-arthritic effects of *Eucommiae Cortex* also reported, as component of mixed herbal formulations[23,24]. *Achyranthis Radix*, a dried root part of *Achyranthes bidentata* Blume, is an important traditional Korean medicine and is extensively used by Korean medicine practitioners for the treatment of osteodynia of the lumbar region and knees, spasm and flaccidity of limbs[25]. In particular, anti-arthritic effects of *Achyranthis Radix* also well-documented through various in vivo and in vitro experiments[26,27]. Since appropriated mixed formulations of medicinal agents have been showed synergic elevation of biological activities on various diseases through diversity of their ingredients[28-32], it therefore, expected that appropriated mixed formulations with dried pomegranate concentrate powders and extracts of *Eucommiae Cortex* and *Achyranthis Radix* will be achieved more potent alternative agents

for osteoarthritis.

II. Materials and methods

1. Primary cultures of normal rat articular chondrocytes

Rat articular chondrocytes for primary culture were isolated from the knee joints of SPF/VAF Outbred Crl:CD1[SD, Spraque-Dawley] male rats (6-week-old upon receipt, body weight ranges 160~180 g; OrientBio, Seungnam, Korea) after 14 days of acclimatization. Cartilage was washed in phosphate-buffered saline (PBS) and finely minced into pieces measuring about 1-3 mm^3. The cartilage tissue was digested for 0.5 h with 0.2% pronase (Sigma-Aldrich, St Louis, MO, USA), and then digested for 4 h with 0.1% collagenase (Sigma-Aldrich, St Louis, MO, USA) at 37°C. After collection of individual cells by brief centrifugation, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic-antimycotic solution (100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B; Life Technologies, Carlsbad, CA, USA). The medium was replaced every 2 days, and the cells were incubated at 37°C under a humidified 5% CO_2 atmosphere. Cells within 5 passage times were used in this experiment. All laboratory animals were treated according to the national regulations of the usage and welfare of laboratory animals by the Institutional Animal Care and Use Committee in Daegu Haany University during acclimatization and sacrifice [Approval No. DHU2014-052].

2. Test materials

The dried pomegranate concentrate powders (PCP; ASYA Meyve Suyu ve Gıda San. A.Ş., Ankara, Turkey) contains 1.15 mg/g ellagic acid as an active ingredient, the *Eucommiae Cortex* aqueous extract (EC) contained 1.62 mg/g pinoresinol diglucoside, the *Achyranthis Radix* aqueous extract (AR) contained 0.25 mg/g ecdysterone, the *Eucommiae Cortex* ethanolic extract (ECe) contained 2.11 mg/g pinoresinol diglucoside and the *Achyranthis Radix* ethanolic extract (ARe) contained 1.06 mg/g ecdysterone (HL Science Co., Ltd., Anyang, Korea). Lipopolysaccharide (LPS; Sigma-Aldrich, St. Louise, MO, USA) and recombinant human interleukin (rhIL)-1α (eBioscience, San Diego, CA, USA) were used to induce the inflammatory response and for degradation and cytotoxicity.

3. Cell viability assay of rat articular chondrocytes

Primary cultured rat articular chondrocytes were seeded in 24-well plates at a density of 2×10^4 cells/well and incubated in a CO_2 incubator at 37°C with 24-h samples (PCP, EC, AR, ECe and ARe at 10 mg/ml concentration) with/without 5 ng/ml rhIL-1α (at 1 h after test substance treatment) before being treated with 100 μl of 2.5 mg/ml MTT (Sigma-Aldrich, St. Louise, MO, USA). Then, the cells were incubated at 37°C for an additional 4 h. After checking production of MTT formazan, 1 ml of DMSO was added, and absorbance was read at 570 nm using a microplate reader (Sunrise; Tecan, Männedorf, Switzerland).

4. Detection of PGE_2 level and 5-LPO and MMP activities in chondrocytes

Primary cultured rat articular chondrocytes were seeded in 24-well plates at a density of 2×10^4 cells/well. The cells were grown in a CO_2 incubator at 37°C with 24-h samples (PCP, EC, AR, ECe and ARe at 1 mg/ml concentration) containing 50 μg/ml LPS (for PGE_2 production and 5-LPO activity; at 1 h after test substance treatment)

or 5 ng/ml rhIL-1α (for MMP activities; at 1 h after test substance treatment). PGE_2 level and 5-LPO, MMP-2, and MMP-9 activities in the supernatants were measured using the PGE_2 assay kit (R&D Systems, Minneapolis, MN, USA), the Lipoxygenase Inhibitor Screening Assay kit (Cayman Chemical, Ann Arbor, MI, USA) and a MMP enzyme-linked immunosorbent assay kit (Mybiosource, San Diego, CA, USA) with a micro-plate reader (Sunrise; Tecan, Männedorf, Switzerland) according to the manufacturer's protocol, as reported previously[12].

5. mRNA expression of extracellular matrix (ECM)-related chondrogenic genes in chondrocytes

Primary cultured rat articular chondrocytes were seeded in 24-well plates at a density of 5×10^6 cells/well. The cells were incubated in a CO_2 incubator at 37°C with 24-h samples (PCP, EC, AR, ECe and ARe at 1 mg/ml concentration) containing 5 ng/ml rhI-1α (at 1 h after test substance treatment). Collagen type II, SOX9, and aggrecan mRNA expression levels were detected on the harvested cells by reverse transcription-polymerase chain reaction analysis according to previously established methods[11,12]. Briefly, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentrations and quality were determined with the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The analysis was carried out using the ABI Step One Plus Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and expression levels were calculated relative to those of the vehicle control. Expression of β-actin mRNA was used as a control for tissue integrity in all samples. The sequences of the PCR oligonucleotide primers were as follows: 1) Collagen type II, F: 5'-GAG-TGGAAGAGCGGAGACTACTG-3', and R: 5'-CT-CCATGTTGCAGAAGACTTTCA-3'; 2) SOX9, F: 5'-AGAGCGTTGCTCGGAACTGT-3', and R: 5'-TC-CTGGACCGAAACTGGTAAA-3'; 3) Aggrecan, F: 5'-GATGTCCCCTGCAATTACCA-3', and R: 5'-TCTGTGCAAGTGATTCGAGG-3'; 4) β-actin, F: 5'-ATCGTGGGCCGCCCTAGGCA-3', and R: 5'-TGGCCTTAGGGTTCAGAGGGG-3'.

6. Statistical analyses

All data were expressed as mean ± SD of the 6 independent experiments. Multiple comparison tests were conducted for the group receiving different doses. The homogeneity of variance was examined using the Levene test. When the Levene test indicated no significant deviations from the homogeneity of the variance, the data were analyzed by one-way analysis of variance followed by the least-significant difference multi-comparison test to identify the significantly different member of a pair. The non-parametric Kruskal-Wallis H test was performed on data with a heterogeneous variance. When a significant difference was detected by the Kruskal-Wallis H test, the Mann-Whitney U-test with Bonferroni's correction was used to determine the specific pairs that differed. Statistical analyses were conducted using SPSS for Windows (Ver. 14.0 Korean edition, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

III. Results

1. Cytotoxic and cytoprotective effects of PCP, EC, and AR on normal primary cultured rat articular chondrocytes

Significant decreases in cell viability were detected in ECe- and ARe-treated chondrocytes at 10 mg/ml, but no changes were demonstrated in PCP-, EC-, or 10 mg/ml AR-treated cells (Fig. 1). Cell viability was decreased significantly in rats treated with 5 ng/ml rhIL-1α compared to the untreated vehicle controls, but rhIL-1α treatment-related cell death was significantly inhibited by pretreatment with all five test substances (Fig. 2).

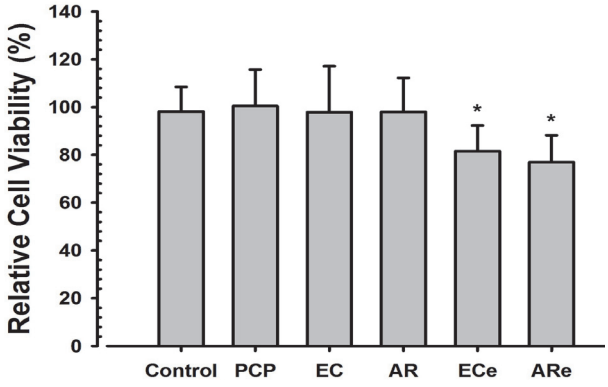

Figure 1. Effects on the normal primary cultured rat articular chondrocyte viabilities. Significant decreases of cell viabilities were detected in ECe and ARe treated chondrocytes at a concentration level of 10 mg/ml, but no meaningful changes on the cell viabilities were demonstrated in PCP, EC and AR 10 mg/ml concentration treated cells as compared with non-treated vehicle control after 24 h after incubation time. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder. * $p < 0.05$ as compared with control.

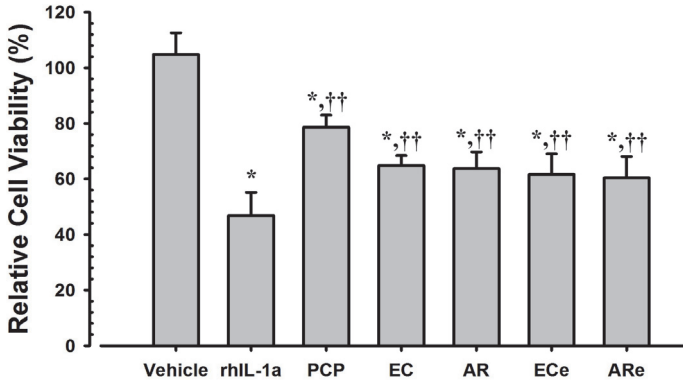

Figure 2. Cytoprotection effect on rhIL-1α-treated chondrocytes. Significant decreases of cell viabilities were detected after treatment of rhIL-1α 5 ng/ml as compared with non-treated vehicle control, but these rhIL-1α treatment related cell deaths were significantly inhibited by pretreatment of all five test substances, in order of PCP > EC > AR > ECe > ARe. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; rhIL = Recombinant human interleukin. * $p < 0.05$ as compared with vehicle control; †† $p < 0.01$ as compared with rhIL-1α treated control.

2. Inhibitory effects of PCP, EC, and AR on PGE2 productions

Marked and significant elevations of PGE_2 contents were observed after treatment of LPS 50 μg/ml as compared with non-treated vehicle control, but these LPS treatment related PGE_2 production elevations were significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR(Fig 3).

3. Inhibitory effects of PCP, EC, and AR on 5-LPO activities

Significant increases of 5-LPO activities were observed after treatment of LPS 50 μg/ml as

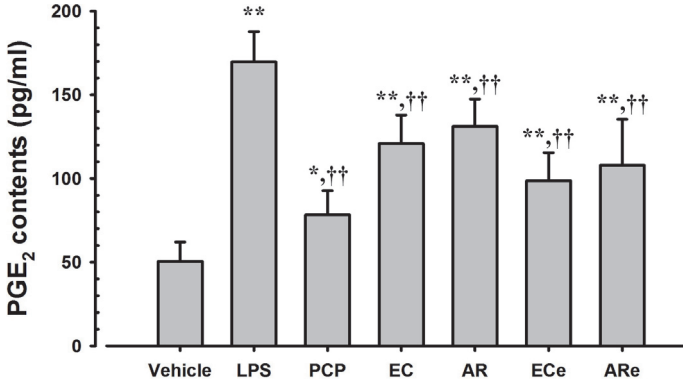

Figure 3. Inhibitory effects on PGE_2 productions. Marked and significant elevations of PGE2 contents were observed after treatment of LPS 50 μg/ml as compared with non-treated vehicle control, but these LPS treatment related PGE2 production elevations were significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; PGE_2 = Prostaglandin E_2; LPS = Lipopolysaccharide. ** $p < 0.01$ and * $p < 0.05$ as compared with vehicle control; †† $p < 0.01$ as compared with LPS treated control.

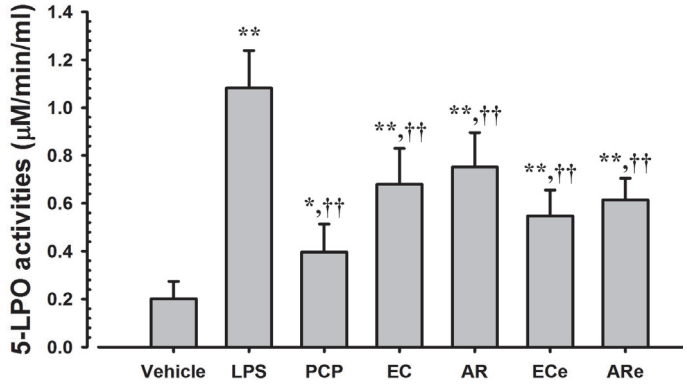

Figure 4. Inhibitory effects on 5-LPO activities. Significant increases of 5-LPO activities were observed after treatment of LPS 50 μg/ml as compared with non-treated vehicle control, but these LPS treatment related increases of 5-LPO activities were also significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; LPO = Lipoxygenase; LPS = Lipopolysaccharide. ** $p < 0.01$ and * $p < 0.05$ as compared with vehicle control; †† $p < 0.01$ as compared with LPS treated control.

compared with non-treated vehicle control, but these LPS treatment related increases of 5-LPO activities were also significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR, in the current experiment (Fig 4).

4. Inhibitory effects of PCP, EC, and AR on MMP-2 and MMP-9 activities

MMP-2 and MMP-9 activities increased significantly in rats treated with 5 ng/ml rhIL-1 α compared with in untreated vehicle controls, but these increases in MMP-2 and MMP-9 activities were also significantly inhibited by pretreatment with all five test substances in the order of PCP > ECe > ARe > EC > AR (Fig 5).

5. Up-regulation ECM related chondrogenic gene mRNA expressions

The mRNA expression levels of ECM-related chondrogenic genes, such as collagen type II, SOX9, and aggrecan, decreased significantly in rats treated with 5 ng/ml rhIL-1α compared with in untreated vehicle controls, but mRNA expre-ssion was significantly upregulated after pret-reatment with all five test substances in the order of PCP > ECe > Are > EC > AR (Fig 6).

IV. Discussion

In this experiment, ECe and ARe showed obvious cytotoxicity against primary cultured rat articular chondrocytes at a dose level of 10 mg/ml. However, no obvious cytotoxic effects of PCP, EC and AR were demonstrated at a dose level of 10 mg/ml. In addition, similar to those of previous reports by other investigators[11,12], treatment of LPS 50 μg/ml induced significant increases of PGE_2 contents and 5-LPO activities indicating infla-mmatory responses of the primary cultured rat articular chondrocytes, and also decreases of cell viabilities, increases of MMP-2 and MMP-9 activities with decreases of ECM related collagen type II, SOX9 and aggrecan mRNA expressions were observed by treatment of rhIL-1α 50 ng/ml,

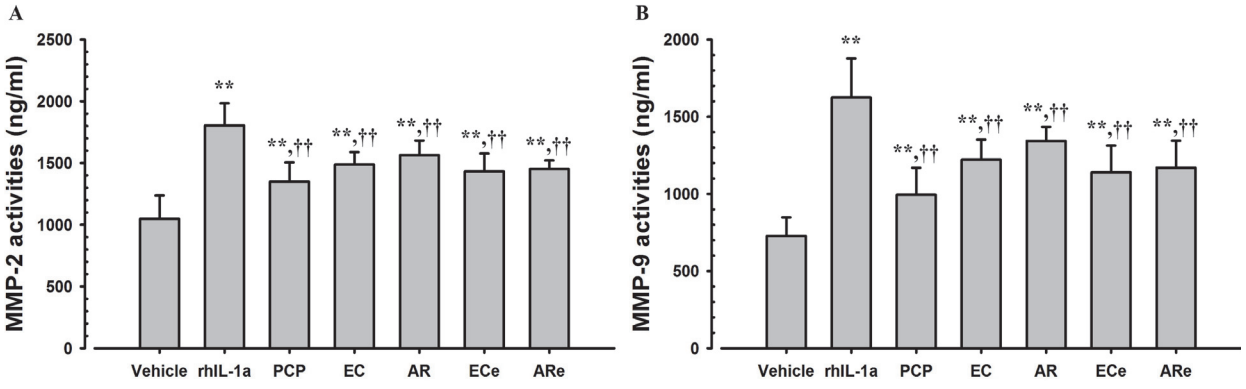

Figure 5. Inhibitory effects on (A) MMP-2 and (B) MMP-9 activities. Dramatic increases of MMP-2 and MMP-9 activities were observed after treatment of rhIL-1α 5 ng/ml as compared with non-treated vehicle control, but these rhIL-1α treatment related increases of MMP-2 and MMP-9 activities were also significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; MMP = Matrix metalloproteinase; rhIL = Recombinant human interleukin. ** $p < 0.01$ as compared with vehicle control; †† $p < 0.01$ as compared with rhIL-1α treated control.

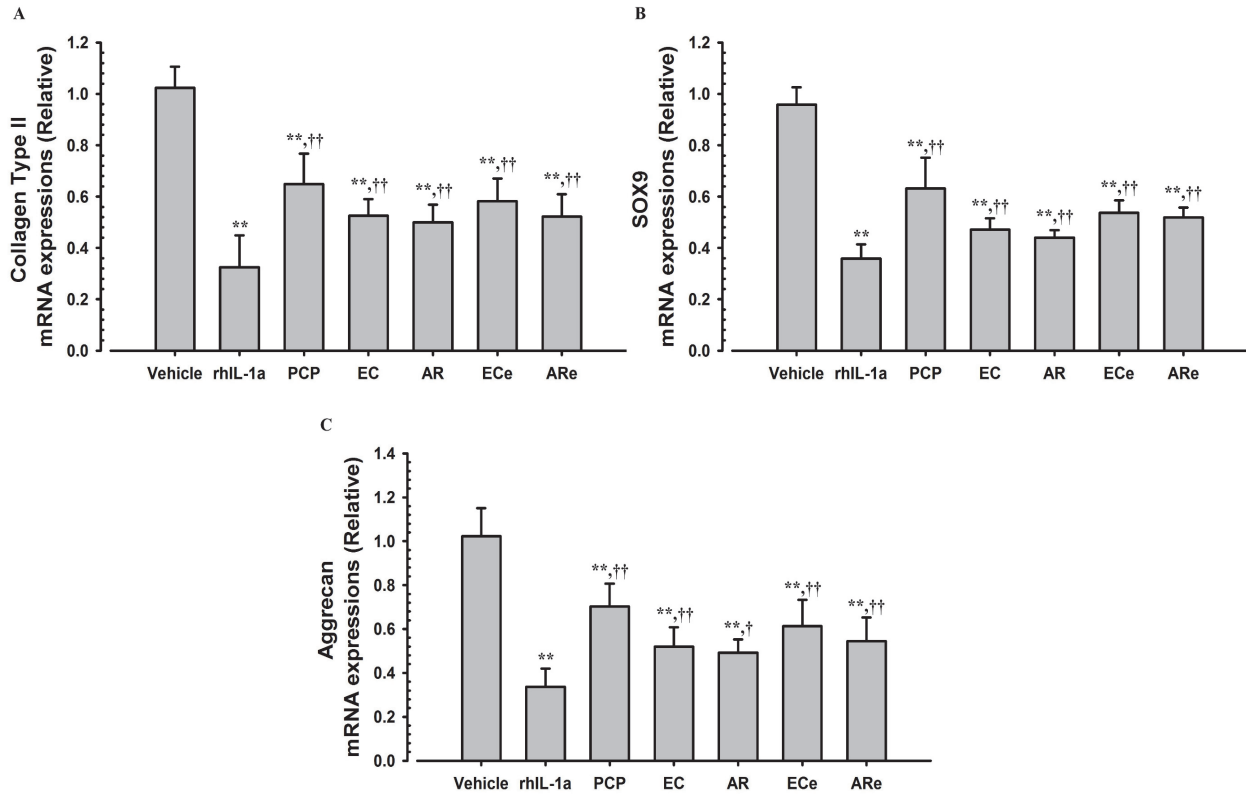

Figure 6. Effects on (A) Collagen Type II, (B) SOX9, and (C) aggrecan mRNA expressions. Noticeable and significant decreases of collagen type II, SOX9, and aggrecan mRNA expressions were observed after treatment of rhIL-1α 5 ng/ml as compared with non-treated vehicle control, but these decreases of mRNA expressions were significantly up-regulated by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR. Values are expressed mean ± SD of six independent experiments. AR = Aqueous extracts of *Achyranthis Radix*; ARe = Ethanolic extracts of *Achyranthis Radix*; EC = Aqueous extracts of *Eucommiae Cortex*; ECe = Ethanolic extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; rhIL = Recombinant human interleukin. ** $p < 0.01$ as compared with vehicle control; †† $p < 0.01$ and † $p < 0.05$ as compared with rhIL-1α treated control.

suggesting damages on the primary cultured rat articular chondrocytes and related ECM degradations. However, these inflammatory damages of chondrocytes and related ECM degradations induced by treatment of LPS or rhIL-1α were inhibited by pretreatment of all five test substance at a dose level of 1 mg/ml, in order of PCP > ECe > ARe > EC > AR, except for cell viability; rhIL-1α induced chondrocytes deaths are inhibited by treatment of PCP > EC > AR > ECe > ARe in that orders.

In MTT assay, favorable osteoarthritis control agent should be showed lower cytotoxic effects on normal chondrocytes, and can be protect the physical and inflammatory response related chondrocyte deaths and damages[11,12]. In the present study, significant decreases of cell viabilities were detected in ECe and ARe treated chondrocytes at a concentration level of 10 mg/ml, but no meaningful changes on the cell viabilities were demonstrated in PCP, EC and AR 10 mg/ml concentration treated cells as compared with non-treated vehicle control after 24 hrs after incubation time. In addition, rhIL-1α treatment related cell deaths were significantly inhibited by pretreatment of all five test substances, in order of PCP > EC > AR > ECe > ARe. These are considered that all test substances have favorable

cytoprotection effects on the chondrocyte damages induced by inflammatory stresses.

Metabolism of arachidonic acid has been regarded as critical mechanisms that regulated the body homeostasis, as part of inflammatory processes; it involved in the synthesis of prostaglandins - chemical mediator of inflammation and leukotriene - chemical mediator to regulate immune responses including inflammatory, asthmatic and allergic responses[33]. 5-LPO is an enzyme that involved in biosynthesis of leukotriene[34], and PGE_2 was produced by other enzyme, cyclooxygenase (COX)-2 coordination with IL-1 and tumor necrosis factor (TNF)-α and involved in etiopathogesis of osteoarthritis, the degradation of articular cartilages[35,36]. In addition, PGE_2 also induced abnormal bone growth and painful osteophytes, which were frequently encounted in the patients suffering from osteoarthritis[12,37]. In the present study, anti-inflammatory potentials of test substances were observed through PGE_2 productions and 5-LPO activities on the LPS treated primary cultured rat articular chondrocytes.

Important enzymes involved in deconstruction of chondrocyte and bony tissue, are proteolytic enzymes, MMPs, and they were inhibited by tissue inhibitor of metalloproteinases system at normal conditions[38]. Since MMPs act as protease that degraded the ECM, mainly PGs[39], they have been regarded as a valuable treatment target in osteoarthritis[11,40]. As results of the present study, the rhIL-1α treatment related increases of MMP-2 and MMP-9 activities were also significantly inhibited by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR, suggesting their powerful MMP-2 and MMP-9 inhibitory effects, and related chondrocyte protective effects, at least in a part of the possibilities of anti-arthritic effects.

It has been believed that degradations of ECM such as collagen type II, aggrecan and SOX9 from the results of inflammation related chondrocyte deaths and damages are involved in the deterioration of the homeostasis and articular functional limitations in osteoarthritis[41]. In this study, the decreases of collagen type II, SOX9 and aggrecan mRNA expressions, induced by rhIL-1α treatment, were significantly up-regulated by pretreatment of all five test substances, in order of PCP > ECe > ARe > EC > AR. These findings are considered that all test materials have favorable articular cartilage preserve effects, through enhancement of ECM production of chondrocytes.

Since this study was conducted under *in vitro* conditions, it is a limitation that these research results are not applied to *in vivo* condition directly, so additional researches are needed to evaluate the efficacy in animal disease model.

V. Conclusion

Taken together the results of this experiment, it is expected that mixed formulation of PCP as main components with appropriate proportion of EC and AR as additional components will be achieved a potent medicinal food for osteoarthritis, through further animal experiments.

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