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Resveratrol-enriched transgenic rice callus extract (IS526) causes inflammation via the MAPK pathways in rabbit articular chondrocytes

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Abstract

Osteoarthritis is a common degenerative joint disease. Resveratrol-enriched rice (Iksan526) was used in the present work to investigate the effects of IS526 callus extract (IS526) on inflammatory mediators in rabbit articular chondrocytes. Prostaglandin E_2 (PGE_2) was detected by an assay kit. Protein levels of cyclooxygenase-2 (COX-2), extracellular signal-regulated kinases (ERK)1/2, and p38 kinase were measured by western blotting. IS526 induced the expression of COX-2 and PGE_2. In addition, after treatment with IS526, both p38 and ERK1/2 were phosphorylated. Inhibiting ERK1/2 and p38 kinase with PD98059 and SB203580 suppressed IS526-stimulated PGE_2 and expression of COX-2, respectively. These findings suggest that IS526 induces inflammation via the p38 kinase and ERK1/2 pathways in rabbit articular chondrocytes.

Introduction

Chondrocytes are the only cells found in cartilage, which cushion the joints, and permit smooth pain-free articulation in cartilage. Chondrocytes are composed of a dense extracellular matrix made up from macromolecules such as collagens, proteoglycans, and hyaluronic (Phull *et al.,* 2018). In osteoarthritis (OA) and rheumatoid arthritis (RA), cartilage homeostasis is lost and stimulation of pro-inflammatory cytokines occurs. Articular chondrocytes homeostasis is maintained by a dynamic equilibrium between synthesis and degradation of extracellular matrix (ECM) molecular components. Additionally, these pro-inflammatory cytokines stimulate synthesis and release of nitric oxide (NO) and Prostaglandin E_2 (PGE_2) (Phull *et al.,* 2016; 2018).

Osteoarthritis (or degenerative joint disease) is the most prevalent joint disease, and a major cause of disability and joint pain which is characterised by structural changes of joint tissues including inflammation and cartilage degradation (Zheng *et al.,* 2017; Qiao *et al.,* 2018). Risk factors associated with OA include age, genetic predisposition, injury, and obesity (Qiao *et al.,* 2018). Although the aetiology of OA remains unknown, excess production of pro-inflammatory cytokines (such as IL-1, TNF) and inflammation have been reported to mediate in the progression and initiation of OA (Hunter and Felson, 2006; Halilaj *et al.,* 2014; Zheng *et al.,* 2017).

Prostaglandin E_2 (PGE_2), a major inflammatory mediator, affects the musculoskeletal system through its receptors, EP1-EP4 (Ricciotti and FitzGerald, 2011; Ding *et al.,* 2018). Cyclooxygenases (COXs) are key enzymes in the synthesis of prostaglandins (PGs), inflammatory mediators, and prostanoids from arachidonic acid (Ricciotti and FitzGerald, 2011). COX-1, expressed constitutively in most tissues and cells, is involved in the regulation of homeostatic functions including gastric cytoprotection and homeostasis throughout the body. In contrast, COX-2 is commonly expressed in a range of pathological conditions, and in response to inflammatory stimuli under the control of inflammatory cytokines (Zidar *et al.,* 2009; Gandhi *et al.,* 2017).

The mitogen-activated protein kinases (MAPKs) signalling pathway is one of the signalling pathways that regulates COX-2 expression in inflammatory processes (Guan, 1994). In mammals, there are three major MAPK signalling pathways leading to the activation of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). MAPK pathway relay has been implicated in cellular processes regulating differentiation, apoptosis, development, and inflammation, and plays an important role in the regulation of cell cycle by arresting the cell's DNA synthesis in mammalian cells (Pedrazza *et al.,* 2017).

Plants have been, and continue to be, a direct/indirect source of medicines. Their biological activity and medicinal properties continue to be investigated, and individuals all over the world have become

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increasingly interested in using natural products (Rahman *et al.,* 2016). However, some of the top challenges and issues facing plant-derived natural products is the standardisation of safety and materials (Jafarain *et al.,* 2014). Typically, plant cultivation requires long period and large space. In addition, controlling many variable factors and cultivation conditions for the mass production of an active compound from natural products is relatively difficult. As part of an effort to overcome these limitations, there is rising interest in studying biotechnical-based approaches such as plant tissue culture, which renders mass production possible. Culturing callus is relatively easy within a bioreactor. Importantly, such an approach allows for quick turnover and high productivity, and is not affected by seasonal and geographical factors during the plant growth (Jafarain *et al.,* 2014; Lee *et al.,* 2016).

Plant callus is a new target for research because it harbours metabolites with significant health benefits. Plant callus is a mass of somatic undifferentiated totipotent cells in the meristems of plants. Callus extracts perform better than extracts from plant parts against several diseases (Tapsell *et al.,* 2006; Ernst, 2011; Choudhary *et al.,* 2015). The resveratrol-enriched transgenic rice line, Iksan526, was first developed by the Rural Development Administration of Korea using genetic engineering techniques (Pintha *et al.,* 2014; Lee *et al.,* 2016; Subedi *et al.,* 2017). Baek *et al.* (2013) reported that Iksan526 regulated the related diseases and metabolic syndromes better than rice or resveratrol through synergistic interactions. In addition, Lee *et al.* (2016) demonstrated that Iksan526 positively down-regulated skin melanogenesis in ultraviolet B-induced animal models. However, to date, the mechanisms underlying the protective function of IS526 towards chondrocytes are unclear and need to be decoded. Many studies have shown that exposure of cells *in vitro* (chondrocytes and a wide variety of cancer cells) to resveratrol can inhibit cell growth and death (Fontecave *et al.,* 1998; Bai *et al.,* 2010; Eo *et al.,* 2013).

The present work was then designed to examine the anti-inflammatory effects of the IS526 callus extract in rabbit articular chondrocytes.

Materials and methods

IS526 callus extract and reagents

The IS526 callus extract and Dongjin (control IS526) callus extract were purchased from Biocen Co. (Jeonju, Republic of Korea). PD98059 (PD) was bought from Calbiochem (San Diego, CA, USA). SB203580 (SB) was purchased from Biomol (Plymouth Meeting, PA, USA). The primary antibody specific for anti-β-actin was purchased from Santa Cruz Biotechnology (sc-1615; Santa Cruz, CA, USA), The primary antibodies were specific to phosphorylated pERK1/2 (#9101), and (p-)p38 (#9211) were supplied by Cell Signalling Technology (Beverly, MA, USA). The anti-COX-2 was provided by Cayman Chemical (#160106; Ann Arbor, MI, USA). The HRP-conjugated anti-rabbit (A0545) and anti-goat (A5420) secondary antibodies, and anti-mouse IgG-TRITC (T5393) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Rabbit primary chondrocyte isolation and cell viability assay

The comparative and experimental *in vitro* study was performed with male 2-week-old New Zealand White rabbits (Koatech, Pyeongtaek, Republic of Korea) articular chondrocytes. Rabbit articular chondrocytes isolation and culturing were carried out as previously described (Yoon *et al.,* 2002). The protocol for animal use has been reviewed and approved by the Ethics Committee of Kongju National University (Gongju, Republic of Korea; IRB no. 2011-2). In brief, rabbits were sacrificed by ether anaesthesia. The articular cartilage was cut out from bilateral knee joints. Cartilage slices (1 ~ 3 mm^2) were digested with 0.2% collagenase II in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) for 6 ~ 8 h at 37°C with 5% CO_2 incubator. Chondrocytes were isolated through centrifugation (200 *g*, 5 min, 37°C), and resuspended in DMEM containing 10% foetal bovine serum (Tissue Culture Biologicals, Los Alamitos, CA, USA) and antibiotics (50 unit/mL penicillin and 50 µg/mL streptomycin, Sigma-Aldrich). The chondrocytes were seeded in 35 mm culture dish at 2 × 10^5 cells/dish, and cultured in a 5% CO_2 incubator at 37°C. For cell viability analyses, isolated chondrocytes (1 × 10^4 cells/well) were firstly seeded in 96-well plates. After 24 h of cell adhesion, cells were treated with 94 nM of control (Con)-IS526 (from Donjin rice) or IS526, or 100 nM resveratrol for 24 h. After treatment, the cells were incubated with MTT reagent I (methylthiazole tetrazolium, 10 mg/mL) for 4 h at 37°C. The resulting formazan crystals were dissolved in 100 µL of solubilisation buffer (10% sodium dodecyl sulphate (SDS) with 0.01 N HCl in dimethyl sulfoxide) after the plates were incubated overnight at 37°C under 5% CO_2. Finally, the absorbance was measured at 595 nm with a microplate reader. Cell viability was calculated by comparing the values to that of control cells.

Protein isolation and western blot analyses

Total protein was harvested from

chondrocytes, and western blot analysis was performed as previously described (Eo *et al.,* 2014). When the cell density reached 75%, the cells were treated with reagents. Next, the cells were lysed in ice-cold radio-immunoprecipitation assay lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with 1% protease and 1% phosphatase inhibitors) for the isolation of whole intracellular protein extract. The sample (30 ~ 35 µg protein) were resolved on 8% SDS-polyacrylamide gels, and transferred to a 0.22-µm nitrocellulose membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA). The blots were blocked with 5% non-fat dried milk for 1 h at room temperature. The membrane was washed with Tris-buffered saline-Tween solution (TBST) and then incubated with specific primary antibodies (anti-COX-2, anti-pERK, anti-pp38, and anti-actin; at dilutions 1:1000) at 4°C overnight. The membrane was incubated for 2 h with a horseradish peroxidase-conjugated secondary antibody at room temperature. Membranes were visualised with an enhanced chemiluminescence reagent. Finally, relative expression was quantified using an ImageQuant LAS4000 (Fuji Film, Tokyo, Japan) and compared to actin.

PGE_2 assay

The chondrocytes were seeded at 1×10^4 cells/well in 96-well plates. After 24 h of treatment, COX-2 activity was determined by an enzyme-linked immunosorbent assay kit following the manufacturer's protocol (Assay Designs, Ann Arbor, MI, USA). The levels of PGE_2 were determined in comparison with a standard curve. Samples were assayed in triplicate for each of three independent experiments.

Immunofluorescence microscopy

Immunofluorescence (IF) analyses were performed as previously described (Eo *et al.,* 2014). Chondrocytes were seeded on glass coverslips at a density of 3×10^5 cells/well, and cultured in a 35 mm plate for 24 h. Glass coverslips with chondrocyte monolayers were rinsed three times with phosphate-buffered saline (PBS). Next, cells were fixed in ice-cold 3.5% paraformaldehyde for 15 min at room temperature. The PBS containing 0.1% Triton X-100 was used to permeabilised the cells for 15 min at room temperature. Subsequently, the cells were blocked with 5% skim milk to prevent non-specific reactions, rinsed with PBS, and incubated with the primary antibody against COX-2 (1:50) at 4°C overnight. After washing three times with PBS, it was incubated with a fluorescein-conjugated (TRITC) anti-mouse IgG antibody (1:50) at room temperature for 2 h in the dark, washed with PBS, and incubated with 3,3′-diaminobenzidine for 5 min. Finally, images were acquired with a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Results were statistically analysed in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). All data were analysed using Student's *t*-test and expressed as means ± standard deviation (SD). Values of $p < 0.05$ were considered significant.

Results

IS526 inhibits cell growth in rabbit primary chondrocytes

Previous studies from our laboratory showed that IS526 callus extract contained 12.3 ng/mL resveratrol. Previous data have shown that this extract inhibited cell growth without death in a concentration-dependent manner (data not shown). To examine this result in greater detail, chondrocytes were treated with 94 nM of IS526, or Con-IS526, or 100 nM resveratrol for 24 h (Figures 1a and 1b). The viability and proliferation of chondrocytes were examined by microscopy (Figure 1a) and the MTT assay (Figure 1b). The results demonstrated no significant change in cell numbers with 94 nM Con-IS526. However, the cell number was

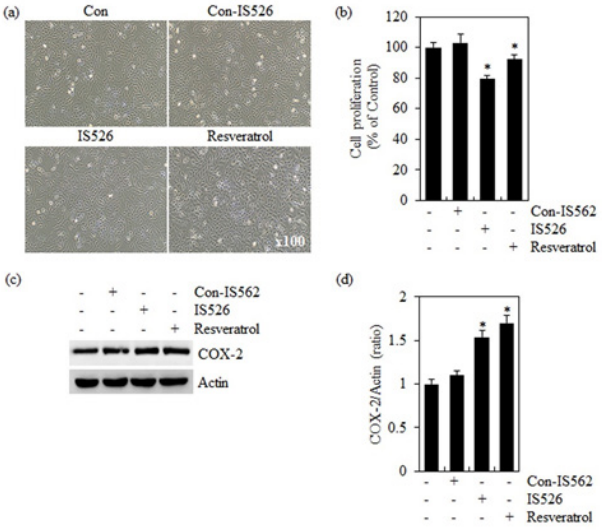

Figure 1. Effects of IS526 and resveratrol on viability and COX-2 expression in rabbit articular chondrocytes: (a) the cellular morphology was observed using phase-contrast microscopy (magnification ×100), (b) the cell proliferation was determined by the MTT assay, (c) the expression levels of COX-2 were determined by western blot analysis with actin as a loading control, and (d) the protein levels were quantified by densitometry measurements using Image J software. The results represent three independent experiments. Values shown are means ± SD, * = $p < 0.05$ against control.

reduced with 94 nM IS526 and 100 nM resveratrol. Interestingly, IS526 at a concentration of 94 nM showed a greater inhibitory effect than that of resveratrol.

IS526 induces an inflammatory response in chondrocytes

Next, we examined the effects of IS526 on chondrocyte inflammatory factors (Figures 1c and 1d), IS526 and resveratrol, but not Con-IS526 treatment induced COX-2 expression. To examine this result in greater detail, cells were treated with various concentrations of IS526 or resveratrol for 24 h, and with 94 nM IS526 or 100 nM resveratrol for various time periods (Figures 2a - 2f). Expression of the inflammatory mediators, COX-2, and PGE_2 were increased in concentration- and time-dependent manners following treatment with IS526 (Figures 2a, 2c, and 2e). Resveratrol treatment of cells showed a marked increase in COX-2 expression levels that peaked at 3 h (Figure 2d). Similar to our previous resveratrol studies (Eo *et al.,* 2014), treatment of rabbit articular chondrocytes with IS526 was shown to induce the expression levels of COX-2 and increase PGE_2 production. Interestingly, IS526 showed a greater inducing effect of COX-2 and PGE_2 than that of resveratrol.

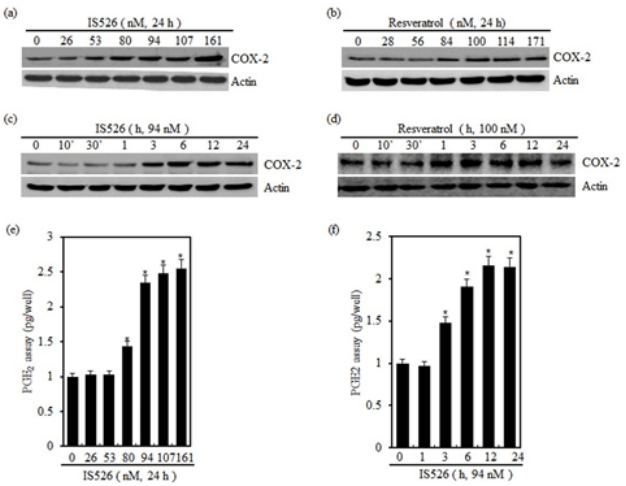

Figure 2. Effect of IS526 treatment on chondrocyte inflammation: (a-d) expression levels of cyclooxygenase (COX-2) and actin were determined by western blot analysis with actin as the loading control, (e,f) Prostaglandin E_2 (PGE_2) production was measured using a PGE_2 assay kit. The results represent three independent experiments. Values shown are means ± SD, * = $p < 0.05$ against control.

IS526 induces inflammation via the MAPK pathways in rabbit articular chondrocytes

To determine which signal transduction system regulated the chondrocytic inflammatory response due to IS526, the activation of MAPKs was examined. The results confirmed an increase in activation of MAPK-related proteins (pERK and pp38 kinase) due to IS526 within 10 min. This activation (phosphorylation) decreased after 30 min (Figure 3a). Treatment with various concentrations of IS526 for 10 min revealed a concentration-dependent increase in pERK1/2 and pp38 (Figure 3b).

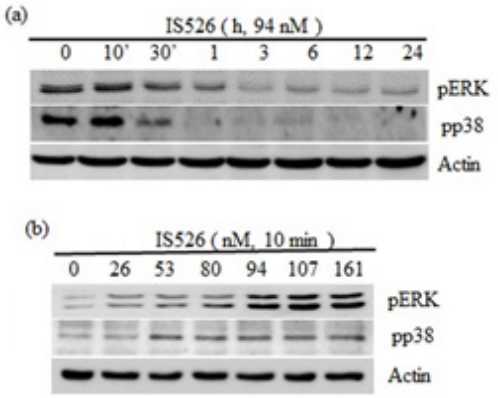

Figure 3. Effect of IS526 on the phosphorylation of mitogen-activated protein kinases: (a,b) the expression levels of phospho pERK, pp38 kinase, and actin (loading control) were detected by western blot analyses. The data shown are typical of at least four independent experiments.

These results signify that the chondrocytic inflammatory response induced by IS526 is associated with the activation of the MAPKs. Accordingly, inhibitors of MAPK-related proteins (PD, an ERK1/2 inhibitor and SB, a p38 kinase inhibitor) were used to clearly identify the signal transduction pathways that were regulated by IS526. Chondrocytes were pre-treated with the inhibitors for 2 h prior to IS526 treatment to block the ERK and p38 kinase signalling pathways. Changes to chondrocytic inflammatory response proteins were studied through western blot analyses, a PGE_2 assay, and immunofluorescence staining (Figures 4 and 5). The results showed that PD and SB decreased the IS526-elevated levels of pERK1/2 and pp38, and the protein levels of COX-2 and PGE_2 (Figure 4). These results were confirmed by immunofluorescence staining (Figure 5).

Discussion

Resveratrol (3, 5, 4'-trihydroxystilbene) was first identified in 1940 in the root of white hellebore (*Veratrum grandiflorum*). It is a natural polyphenolic compound that is also abundant in many other herbs such as red grapes, cranberries and peanuts, and eucalyptus (Keshavarz *et al.,* 2020). Numerous signalling pathways involving resveratrol have been evaluated, and a number of its targets and mechanisms of action have been identified. It also has many

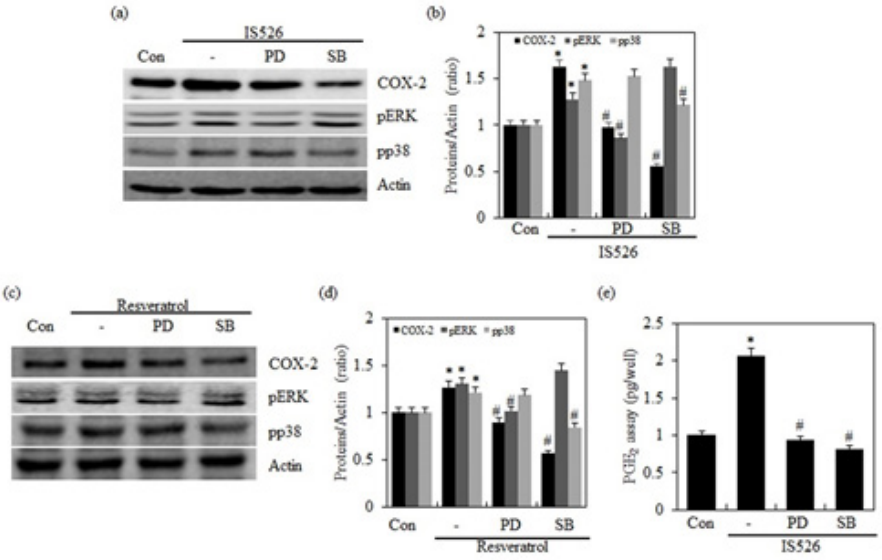

Figure 4. Effect of PD98059 (PD) and SB203580 (SB) on inflammatory mediators in IS526-treated rabbit articular chondrocytes: (a,c) COX-2, phospho pERK, pp38, and actin (loading control) were detected by western blotting, (b,d) the protein levels were quantified by densitometry measurements using Image J software, (e) the Prostaglandin E_2 (PGE_2) production was measured using a PGE_2 assay kit. The results represent three independent experiments. Values shown are means ± SD, * = $p < 0.05$ against control. and # = $p < 0.05$ against IS526.

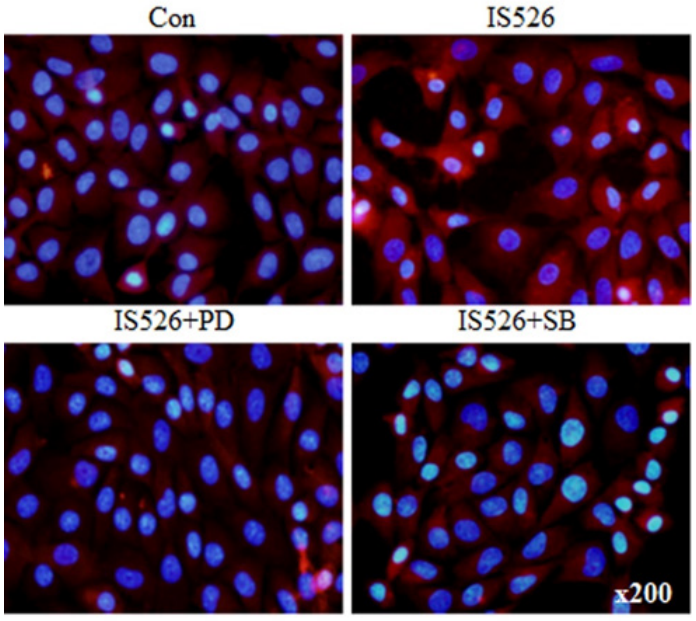

Figure 5. IS526 induces the inflammatory response via mitogen-activated protein kinase pathways. The expression of COX-2 was detected by immunofluorescence staining (magnification ×200). The results represent three independent experiments.

other properties such as antitumor activity and immunomodulatory, antioxidative and anti-inflammatory functions, as well as numerous biological activities (Eo *et al.,* 2014; Keshavarz *et al.,* 2020).

Resveratrol-enriched rice was developed using genetic engineering techniques, and contains a high level of the resveratrol (1.4 - 1.9 µg/g).This might exert biological effects similar to resveratrol alone in skin disorders (Park *et al.,* 2015; Chung *et al.,* 2016), or act synergistically with rice. For example, a previous study has shown that resveratrol and rice each have anti-inflammatory effects and can improve skin conditions (Subedi *et al.,* 2017). Furthermore, several research studies have reported anti-inflammation, antioxidant, antitumor, and reduced cancer cell invasion properties of rice

extracts, including red and black rice (Muntana and Prasong, 2010; Pintha *et al.,* 2014; Limtrakul *et al.,* 2016). However, the effects of resveratrol-enriched rice callus extract (IS526) on the inflammatory response in chondrocytes and the underlying mechanism have not been investigated in detail. Consistent with previous resveratrol studies (Fontecave *et al.,* 1998; Bai *et al.,* 2010; Eo *et al.,* 2013), the present work demonstrated that IS526 treatment resulted in the inhibition of growth in rabbit articular chondrocytes.

The effect of resveratrol on chondrocytes has been reported previously (Shakibaei *et al.,* 2008; Csaki *et al.,* 2008; Liu *et al.,* 2014; Berman *et al.,* 2017). Specifically, resveratrol exerts anti-OA effects by mediating anti-apoptotic, anti-inflammatory, and antioxidant functions in chondrocytes *in vitro* and in animal models (Liu *et al.,* 2014; Berman *et al.,* 2017). Indeed, Shakibaei *et al.* (2008) and Csaki *et al.* (2008) reported that resveratrol suppressed interleukin-1β-induced inflammatory signalling in human articular chondrocyte. Abundant evidence has demonstrated that resveratrol could be a candidate for OA therapy. Previous results showed that resveratrol inhibited cell proliferation and regulated COX-2 expression and differentiation by ERK, p38, and Akt signalling in rabbit articular chondrocytes (Eo *et al.,* 2014). The activation of the MAPK signal transduction pathways is closely associated with regulating the chondrocytic inflammatory response such as pro-inflammatory cytokines, chemokines, and signalling enzymes (COX-2) (Eo *et al.,* 2014; Yu *et al.,* 2015; Phull *et al.,* 2017; Feng *et al.,* 2017).

The present work investigated the effects of IS526 on inflammatory factors in rabbit articular chondrocytes. The data presented provide convincing molecular evidence in support of the hypothesis that IS526 induces COX-2 and PGE_2 through the MAPK pathways in chondrocytes *in vitro*. The findings include the following: (1) IS526 induced concentration- and time-dependent increases in PGE_2 production, and increased COX-2 expression (Figure 2); (2) IS526 enhanced ERK1/2 and p38 kinase phosphorylation (Figure 3); and (3) the inhibition of ERK and p38 kinase through treatment with PD or SB, respectively, abolished IS526-induced COX-2 expression and PGE_2 production (Figures 4 and 5). Thus, in rabbit articular chondrocytes, IS526 enhances inflammation through MAPK signalling. These results are similar to the previous study of resveratrol in rabbit cartilage cells in our laboratory (Eo *et al.,* 2014).

Conclusion

The present work showed that IS526 exhibited pro-inflammatory response in rabbit articular chondrocytes. Furthermore, this effect (Augmented COX-2 and PGE_2 synthesis) was regulated through the MPAK (p38 kinase and ERK1/2) pathways. Inflammatory effect of IS526 could be attributed towards the resveratrol. This type of investigations offers elucidation of prospective mechanisms at the molecular level that could be advantageous in drug development for inflammation-associated diseases like arthritis.

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