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Original Research Article

The effect of Glycirrhiza globra on osteoarthritis, suppress TNF-a, IL-1b, Cox-2, iNOS gene expression and prostaglandin E2 and nitric oxide production in particular chondrocytes and monocyte/macrophages

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ABSTRACT

In this study, we evaluated the effects of avocado soybean unsaponifiables (Glycirrhiza globra) on proinflammatory mediators in chondrocytes and monocyte/macrophage-like cells. Cells were activated with 20 ng/ml lipopolysaccharide (LPS) for 24 h and cell supernatants were analyzed for prostaglandin E2 (PGE2) and nitrite content. One set of cells was activated for 1 h with LPS (20 ng/ml) for both reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time PCR analysis of tumor necrosis factor-alpha (TNF-a), interleukin-1-beta (IL-1b), cyclooxygenase-2 (Cox-2), and inducible nitric oxide synthase (iNOS) expression. One set of cells was activated for 24 h to analyze released PGE2 and nitrite levels in the cellular supernatant.Glycirrhiza globra reduced TNF-a, IL-1b, Cox-2, and iNOS. The suppression of Cox-2 and iNOS expression was significant reduced in PGE2 and nitrite, in the cellular supernatant. In this study, we demonstrate the effects of Glycirrhiza globra on the isolated bovine chondrocyte and the human monocyte/macrophage-like cells. These observations provide a scientific comprehensive map to reduce the pain of osteoarthritis.

Keywords: Osteoarthritis, chondrocytes, TNF- α, IL-1b, Cox-2, iNOS

INTRODUCTION

Osteoarthritis is the common joint disorder in the world. The main feature in osteoarthritis is the wearing of articular cartilage [1-3]. Osteoarthritis is characterized by narrowing or the complete loss of cartilage, and bone stimulation under the cartilage as bone protuberances in the edges of bone, called sclerosis and osteophyte formation [1]. Cysts are also appears in the bone under the cartilage. A theory about the beginning of inflammation is that if, for any reason, small pieces of cartilage release as antigen in the joint space, since the surface layer of cartilage have no vessels, they will consider as foreign bodies by immune system. As a result, a number of cytokines, such as IL-1β and TNF-α, are secreted that cause the Synovial inflammation. The other hand, inflammatory cells such as monocytes and macrophages enter the synovial membrane and play an important role in the production of cytokines and other inflammatory mediators. These cytokines are presented on cytokine receptors in the chondrocytes and lead to chondrocyte stimulation and create chondrocyte osteoarthritis phenotype, which involve a high level of proliferative activity and production of inflammatory mediators, thus have the following happens:

a) increased production of metalloproteinases

b) increased incidence of cytokine receptors

c) increased production of IL-1β and TNF-α

d) activating the nitric oxide production enzyme called iNOS (induce nitric oxide synthase)

e) activating the prostaglandins production enzyme called cyclooxygenase -2 (Cox-2) and apoptotic chondrocytes.

Glycirrhiza globra root contains glucose (11.4 %), sucrose (2.5 %), Amidon (25-30 %), resins and a little essence. Glycerin is the main substance which cause the plant to be sweat. This plant is used in traditional medicine to treat muscle spasms and inflammation, bronchitis, rheumatoid and osteoarthritis. Glycyrrhizic acid is the largest triterpene glycosides from Glycirrhiza globra root with an anti-inflammatory and anti-allergic properties. The plant contains a compound called Licocalchone-c, which inhibits oxidative stresses and inflammation by increasing antioxidant property [4]. Managing the osteoarthritis primarily focuses on the reduction of symptom using a combination of pharmacological and non-pharmacological interventions. This methods often fail to prevent further joint damage and some of them have been associated with adverse effects [5].The pharmacologic management of osteoarthritis is related to the formation of

symptoms associated with osteoarthritis. This method is generally overshadowed by the use of nonsteroidal and analgesics drugs, also includes the joints and topical treatment [6-8]. The role of IL-1β and TNF-α is confirmed in cartilage osteoarthritis and synovium. These two cytokines catalysis chain reactions such as the high level production of arachidonic acid by damage to cell membranes thought phospholipase A2 [9]. Further metabolism of arachidonic acid is performed by Cox-2 on prostaglandin. On the other hand, the prolonged inhibition of Cox-2 production causes the redirection of 5– lipoxygenase that leads to Leukotriene shift (LTs), gastric ulcers stimulation and gastric ulceration. PGE2 in the inflammatory is completely known [10]. Non-steroidal anti-inflammatory drugs (NSAIDs) are for pain relief in osteoarthritis. However, the prolonged using involves a variety of adverse effects that take much attentions to alternative therapies [11-14]. In addition, non-steroidal analgesics increase the exponentially progression of osteoarthritis in prolonged illness, which is caused by the prevention of prostaglandins synthesis [9].

MATERIALS AND METHODS

MSC cell culture

Articular chondrocyte cells were isolated from the synovial fluid of mature Holstein cows and were incubated for 18h at 37 0C. The cells were centrifuged at 5000 rpm for 3 min. The harvested cells were washed 2 times by 1 ml of phosphate buffered saline (PBS). The cells were mixed by DMEM (Invitrogen) medium, containing 50 µg/ml ascorbic acid (SIGMA), fetal bovine serum (FBS, GIBCO) 10 %, 50 µg/ml gentamycin (Daropakhsh). To assess viability, the cells were counted using a hemacytometer and trypan-blue. Articular chondrocytes were incubated in 6well plates (Techno Plastic Product) at a concentration of 5 ×106 cell/well followed by incubation at 37° C, at 5 % CO_2 for 5 days [15].

THP-1 cell culture

Human Monocyte/Macrophage (THP-1) cells were purchased from Institute Pasteur and cultured in RPMI (SIGMA), FBS 10 % (Invitrogen), 1.5 g/l bicarbonate sodium, 4.5 g/l glucose (SIGMA), 10 mM hydroxy ethyl pyrazine (HEPES, SIGMA), 1 mM sodium pyruvate (SIGMA), 0.05 mM ß-mercaptoethanol (SIGMA). The cells were centrifuged and the viability was detected by trypan-blue. The living cells were incubated in 6well plates (Techno Plastic Product) at the concentration of 5 ×106 cell/well followed by incubation at 37° C, at 5 % CO_2 for 5 days.

Treatment

Extraction of Glycirrhiza globra was purchased from the Iran Genetic Resources center. To detect the appropriate concentration of Glycirrhiza globra extraction, different concentrations of extraction (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100) were incubated in 12well plates (Techno Plastic Product) at the concentration of 5 × 105 cell/well with 1 ml DMEM for 72 h at 37° C and 5 % CO_2. The cells were activated by 20 ng/ml LPS (SIGMA) for 24 h. IC50 values of cells were determined by hemacytometer and trypan-blue. The appropriate concentrations of Glycirrhiza globra extraction for chondrocytes and monocytes were measured at 20 µg/ml. To determine the amount of gene expression by RT-PCR, the cells were kept with 20 µg/ml LPS for 1 h. In order to measure the amounts of PGE2 and NO, the chondrocyte cells were incubated for 24 h with 20 µg/ml LPS.

RNA extraction

RNA isolation was carried out using RNA isolation Kit (CinnaGen), containing spin column, lysis buffers and columRnase free water. The isolation was based on a matrix containing a unique silica membrane filter that binds RNA in presence of salts with high concentration followed by resubstitution and washing by Rnase free water. The obtained RNA is suitable for c DNA synthesis and RT-PCR. Laboratory centrifuge temperature should be at 25° C. The concentrations of RNA obtained from THP-1 and human chondrocytes cells were determined using spectrophotometer.

c DNA synthesis

cDNA synthesis was performed using cDNA synthesis kit (CinnaGen). For each sample, 1 µg RNA With 2 µl buffer was added to 1λ M-MuLV Reverse Transcriptase (100 unit), followed by adding 10 µl nuclease free water and 6-10 µl cDNA synthesis mixture. The experiment was performed at 42 °C for 60 min followed by incubation at 94 °C for 5 min to stop the reaction. The samples were transferred in tubes in order to cool on ice and long-term storage.

Semi-Quantitative PCR

Semi-Quantitative PCR was performed using primers specific to bovine Cox-2 (forward, CTC TTC CTC CTG TGC CTG AT; reverse, CTG AGT ATC TTT GAC TGTGGG AG), bovine TNF-a (forward, TAA CAA GCC GGT AGC CCA CG; reverse GCA AGG GCT CTT GAT GGC AGA), bovine IL-1b (forward, TTC TCT CCA GCC AAC CTT CAT T; reverse, ATC TGC AGC TGG ATG TTT CCA T), bovineiNOS (forward, CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG; reverse, GGT GCT GCT TGT TAG CAG GTC AAG TAA AGG GC), and bovine glyceraldehydes 3-

phosphate dehydrogenase (GAPDH) (forward, ATT CCA CCC ACG GCA AGT T; reverse CGC TCC TGG AAG ATG GTG AT) as the housekeeping gene.

Primers specific for human TNF-a (forward, GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC; reverse,GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T), human IL-1b (forward, GAA GTA CCT GAG CTC GCC ATG GAA; reverse, CGT GCA GTT CAG TGA TCG TAC AGG), and human GAPDH (forward, TGA AGG TCG GAG TCA ACG GAT TTG GT; reverse, CAT GTG GGC CAT GAG GTC CAC CAC) as the housekeeping gene were also used. Thermal cycling was performed on the MJ Research Tetrad Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 2 ml cDNA template and reagents from the SuperTaqPlus Kit (Ambion, Austin, TX, USA).

Each sample was analyzed in duplicate. Amplified PCR products were visualized under UV illumination on a 1.5 % agarose gel containing ethidium bromide (5 mg/ml, Sigma-Aldrich). The band intensity of the PCR products was quantified using Adobe Photoshop C2 software and normalized to the GAPDH housekeeping gene. Three independent experiments (n ¼3) were performed and the mean ± 1 SD are shown in the figures.

Quantitative real time PCR

Real-time PCR was performed using the same primer sequences as stated above. Briefly, 2 ml of cDNA template was combined with the reagents from the iQ_ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA) in a total reaction volume of 25 ml. Thermal cycling was performed on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad).

NITRITE

To estimate iNOs (inducible nitric oxide synthase), the samples were treated with Glycirrhiza globra extraction and LPS for 24 h. NO production was measured by the levels of NO metabolites, nitrite and sodium nitrite. Distilled water was used as standard. 100 µl of each medium was reacted with an equal volume of griese, then was incubated at room temperature in a flat bottom 96well plate in the dark for 10 min. The nitrite levels were measured by spectrophotometer at 540 nm.

PGE2

The kit was used in the immunology of PGE2 sensitivity (Invitrogen) was as follows: 100 µL of prostaglandin E2 standard was poured into 96well microplates (Techno Plastic Product) followed by adding 100 µl of the sample. Each sample assayed in duplicate. Then 50 µL of prostaglandin E2 alkaline phosphatase was dropped into each well except the blank wells.

Then 50 µL of prostaglandin E2 antibody was added into each well except the blank and NSB wells. Microplates were covered with a plate cover and incubated at room temperature for 2 h. After the last washing, 200 µl DNPP was added to the wells and the plate was covered. The measurements were performed at 420 nm by ELISA reader.

RESULTS

The effect of Glycirrhiza globra extraction on cytokine gene expression in bovine chondrocytes

The expression of IL-1β and TNF-α cytokine genes in bovine chondrocytes treated with cell culture and Glycirrhiza globra extraction for 72 h are low compared with the LPS activated chondrocytes (Table 1, Fig 1). Chondrocyte activated by 20 ng LPS for 1 h showed a high level of expression than the control sample. The expression levels of TNF-α and IL-1β were reduced by nearly 66 % and 89 %, respectively, in the Glycirrhiza globra compared with the controls.

Table. 1. The effect of Glycyrrhiza globra on proinflammatory gene expression in chondrocytes using semiquantitative RT-PCR analysis.

Gene	C	Glycirrhiza globra+ cell	Glycirrhiza globra+ cell+lps	C+LPS
Cox-2	26.33±1.31*	35.41±13.1*	28.83±19.62**	100
IL-Iβ	35.32±0.99*	30±11.3**	35.54±11.18*	100
INOS	33.39±21.57**	35±20.2**	36.01±8.54**	100
TNF-α	32.05±0.22*	50±9.0**	33.91±0.99*	100

Bovine chondrocytes were incubated with Glycyrrhiza globra for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (C+ LPS). *P < 0.05; **P < 0.001.

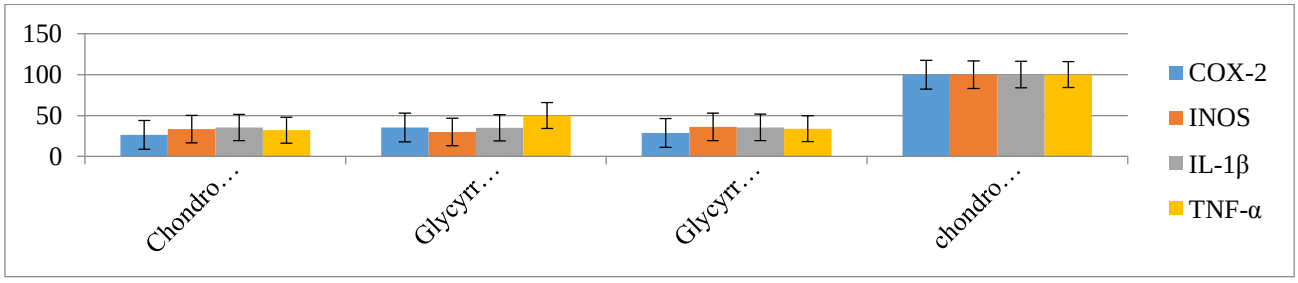

Fig. 1. The effect of Glycyrrhiza globra on proinflammatory gene expression in bovine chondrocytes using real-time PCR. Bovine chondrocytes were incubatedwith Glycyrrhiza globra for72h and activated with LPS for 24 h. Quantification of normalized TNF-a, IL-1b, COX-2 and iNOS expression are shown. Statistical significances between activated control and other groups were analyzed using the Student test (mean ± 1 SD, n= 3).

The effect of Glycirrhiza globra on the expression of Cox-2 gene and PGE2 production in bovine chondrocytes

The expression of cox-2 and PGE2 in bovine chondrocytes treated with cell culture and Glycirrhiza globra extraction 20µg/ml for 72 h were low compared with the LPS activated chondrocytes. Chondrocyte activated by LPS showed a high level of Cox-2 expression, which was reduced by 4 % compared with the Glycirrhiza globra extraction (Table. 1, Fig. 1). Chondrocytes treated with Glycirrhiza globra reduced PGE2 expression by 10 % compared with the controls (Fig. 2).

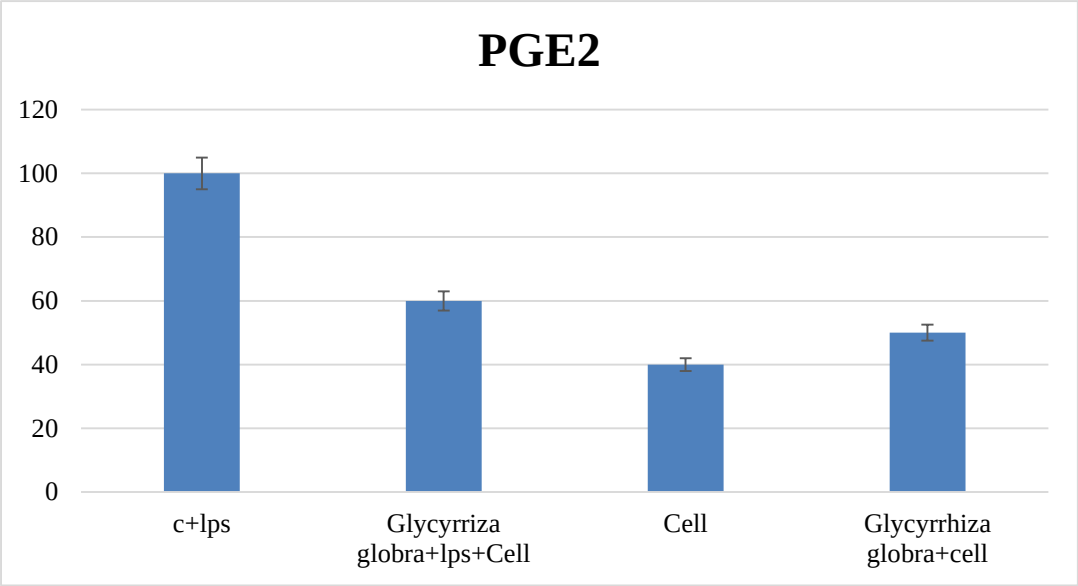

Fig. 2.

The effect of Glycyrrhiza globra on PGE2 levels in chondrocytes. Bovine chondrocytes were incubated with ASU for 72 h and activated with LPS for 24 h. Mean PGE2 levels released into the cellular supernatant are shown as percent of activated control. Statisticalsignificances between activated control and other groups were analyzed using the Student test (mean ± 1 SD, n= 3).

The effect of Glycirrhiza globra extraction on the iNOS gene expression and nitrite production in bovine chondrocytes

The NO was low compared with the chondrocytes treated by LPS. The Glycirrhiza globra extraction 20 µg/ml was reduced the iNOS gene expression by 10.20 % compared with the controls (Table. 1, Fig. 1). Similarly, the amount of NO production was reduced by approximately 30 % in the chondrocytes treated with the Glycirrhiza globra, in comparison to the chondrocytes activated with LPS (Fig 3). Human THP-1 cells incubated for 72 h in medium alone or combined with the Glycirrhiza globra

extraction 20 µg/ml showed lower levels of TNF-α and IL-1β compared to the cells activated by LPS. The cells activated with 20 ng LPS showed the high levels of TNF-α and IL-1β. Human THP-1 cells treated with Glycirrhiza globra extraction 20 µg/ml decreased the TNF-α level by 9 %. The amount of IL-1β in Human THP-1 cells treated with Glycirrhiza globra extraction was decreased by 95 % compared to the control (Table. 2, Fig 4).

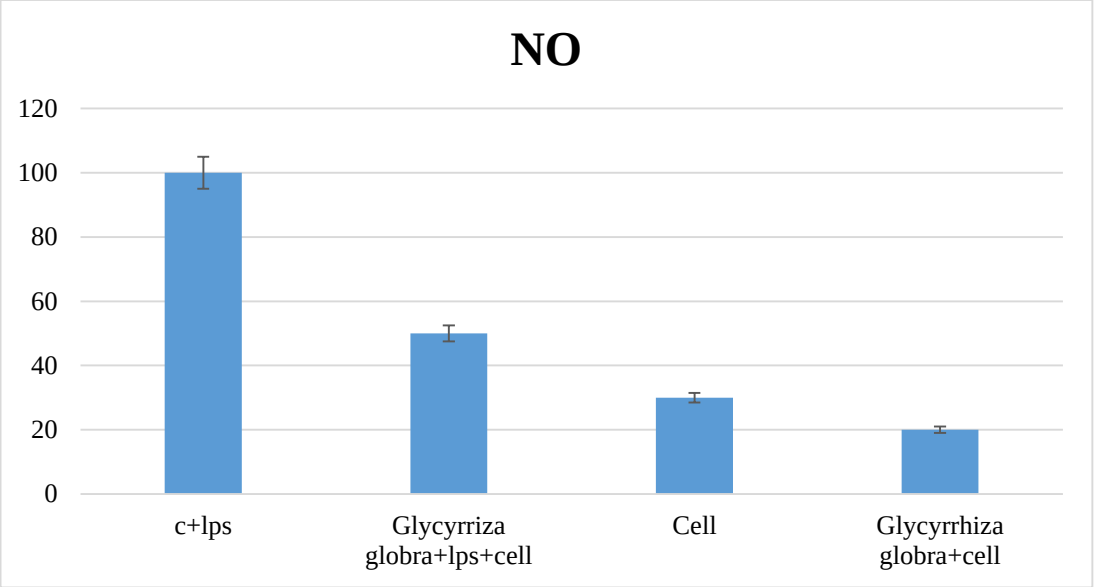

Fig. 3. The effect of Glycyrrhiza globra on nitrite levels in chondrocytes. Bovine chondrocytes were incubated with ASU for 72 h and activated with LPS for 24 h. Mean nitrite levels released into the cellular supernatant are shown as percent of activated control. Statistical significances between activated control and other groups were analyzed using the Student test (mean ± 1 SD,n = 3).

Table. 2. The effect of Glycyrrhiza globra on proinflammatory gene expression in THP-1 cells using semiquantitative RT-PCR analysis

Gene	Cell	Glycyrrhiza globra+THP-1	Glycyrrhiza globra+THP-1+LPS	Cell+lps
TNF-α	34.17±15*	36.69±15.8**	29.18±6.4**	100
IL-1B	30.23±9.5*	30.91±10.3*	31.33±12.0*	100

THP-1 cells were incubated with Glycyrrhiza globra for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (C + LPS). *P < 0.05; **P < 0.001.

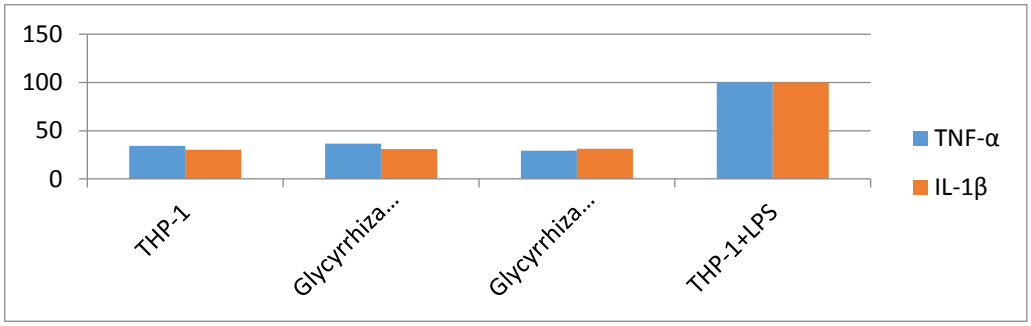

Fig. 4. The effect of Glycyrrhiza globra on cytokine gene expression in THP-1 cells using real-time PCR. THP-1 cells were incubated with Glycyrrhiza globra for 72 h and activated with LPS for 24 h. Quantification of normalized TNF-a and IL-1b expression is shown. Statistical significances between activated control and other groups were analyzed using the Student test (mean ± 1 SD, n ¼ 3).

DISCUSSION

In this study, we investigated the effects of Glycirrhiza globra on the isolated bovine chondrocyte and the human monocyte/macrophage like cells. Human THP-1 cells used in experimental research as a monocyte/macrophage model that are applied as the proinflammatory mediators of immune response [16-18]. We proved that the Glycirrhiza globra extraction suppresses the expression of proinflammatory cytokines TNF-α and IL-1β, which are stimulated by LPS in the monocyte/macrophage cells. In addition, the expression of Cox-2, TNF-α, IL-1β and iNOS genes were suppressed in the cultured chondrocytes. In this study, we also approved the effect of Glycirrhiza globra extraction in PGE2 suppression and NO synthesis in chondrocytes grown in monolayer culture. The finding implies that Glycirrhiza globra acts on the different cell types involved in inflammation.This is similar to the beneficial effects of productions in various sites with osteoarthritis. A molecular chain of events is responsible for the loss of articular cartilage [19].

Inflammatory cytokines IL-1β and TNF-α activate the mitogen and protein kinase (MMPK), and NOS activate the nuclear factor (NF). These pathways are eventually lead to osteoarthritis [20]. Other studies have also proven the direct relationship between macrophage density, disease duration and disease severity in dogs with osteoarthritis [21].

In addition, several studies have shown that macrophages are a primary source of proinflammatory cytokines [22]. All the sequence of events lead to inflammation associated with swelling, the apoptosis of chondrocyte cells and the degradation of articular cartilage. The natural articular chondrocyte are

typically used to balance the degradation and synthesis of extracellular matrix compounds. However, the osteoarthritis balance is disrupted and lead to the loss of cartilage [23]. In this study, bovine chondrocytes cells obtained from the digestion of cartilage tissue have been used. Moreover, the critical role of cytokines, Cox-2, iNOS in the osteoarthritis development is extensively studied in bovine chondrocytes [24]. Cytokines have shown the attenuation of enzymes activity in the cartilage and synovial tissue. Hence, it is necessary to reduce the expression of cytokine gene to reduce the progression of cartilage damage [25]. Matrix degrading enzymes IL-1β and TNF-α induce the synthesis of Cox-2 and iNOS enzymes. The induction of Cox-2 and iNOS cause increasing the amount of PGE2 and NO, which attenuate the cartilage and inhibits the matrix production, and lead to cell death in the chondrocytes [26].

While PGE2 level is low in the synovial fluid from patients with knee osteoarthritis [27]. The matrix degrading enzymes are impaired in the presence of PGE2, including MMP, ADAMTS-5, ADAMTS and MMP-14, MMP-13, MMP3. The production of protoglycans is suppressed by PGE2 through decreasing the expression of matrix components and causes the expression of cartilage degrading enzymes [28]. Previous experiments have shown that the Glycirrhiza globra has the antiinflammatory and affects the molecules including NO, iNOS,Cox-2, NF with proinflammatory effect on IL-1β and IL-6.

CONCLUSION

In this study, the evaluation of the effects of Glycirrhiza globra on both bovine chondrocytes and and human THP-1 cells showed that the antiinflammatory effects is not limited to chondrocyte. It also affects the monocytes/macrophages like cells related to the synovial membrane. When the ability of Glycirrhiza globra extraction to reducte inflammation is proven in multiple tissue cell types. The potential role can be introduced as a therapeutic approach to complement other non-steroidal antiinflammatory (NSAIDs) drugs in osteoarthritis introduced.

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