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Kaempferol Alleviates the Interleukin-1 β -Induced Inflammation in Rat Osteoarthritis Chondrocytes via Suppression of NF- κ B

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Data Interpretation D
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Background: This study was designed to examine the anti-inflammatory and anti-osteoarthritis (OA) effects of kaempferol in rat articular chondrocytes stimulated with interleukin-1 β .





Material/Methods: Rat articular chondrocytes cultures were treated with interleukin-1 β alone or with kaempferol (25, 50, 100, and 200 μ M) and interleukin-1 β . The effect of kaempferol on chondrocyte cells viability was measured by MTT assay. The effect on prostaglandin E2 (PGE2) and nitric oxide (NO) level were also assessed using the ELISA and Griess reagent, respectively, for kaempferol activity. Moreover, the expression of iNOS, Cox-2 and activation of NF- κ B under influence of kaempferol was also assessed by Western blot.

Results: Kaempferol treatment (up to 100 μ M) in a concentration-dependent way caused reduction in the interleukin-1 β -stimulated formations of PGE2 and NO. Kaempferol also upregulated the expression of iNOS and Cox-2 in interleukin-1 β -stimulated rat OA chondrocytes. Additionally, kaempferol was found to inhibit the I κ B α degradation and NF- κ B activation in rat chondrocytes stimulated with interleukin-1 β .

Conclusions: Kaempferol significantly caused reduction in interleukin-1 β -stimulated pro-inflammatory mediators in rat OA chondrocytes by inhibiting the NF- κ B pathway. These results suggest that kaempferol had significant anti-inflammatory and anti-arthritis effects. Thus, kaempferol, as a novel therapeutic active agent, may prevent, stop, or retard the progression of OA.

MeSH Keywords: **Inflammation • Interleukin-1beta • Osteoarthritis**

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Background

Osteoarthritis (OA) is considered a form of arthritis affecting many individuals [1]. Across the globe, OA is the major cause of physical disability with chronic pain in middle-aged to older adults [2,3]. It is a chronic disease generally represented by progressive degeneration of articular cartilage along with the depletion of the cartilage matrix [4,5]. In the United States, about 27 million people (aged of ≥ 25 years) were affected by OA in 2005 [6].

The articular cartilage contains a specific type of cell known as chondrocytes, which usually generate and control the cartilage matrix [7]. Modifications in normal chondrocytes are typically observed in the early and advanced stage of OA development as a result of a greater response to the pro-inflammatory mediators [8,9]. Major pathological features of OA are associated with the gradual degradation of articular cartilage; hence this is usually a target for development of disease-modifying therapy [10]. Overexpression of pro-inflammatory mediators is known to be related to OA progression [11]. Numerous studies have suggested that attenuation of interleukin-1 β (IL-1 β) stimulated inflammatory mediator could be a first-line treatment strategy for OA [12]. The role of higher formations of nitric oxide (NO) and prostaglandin E2 (PGE2) are being studied as major contributors to the development of OA [13,14]. Various studies have documented that stimulation of interleukin-1 β (IL-1 β) may potentially increase the expression of Cox-2 (cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) and consequently leads to a higher production of NO and PGE2 [15,17]. The NF- κ B signaling pathway is thought to be associated with interleukin-1 β stimulated OA. The pro-inflammatory responses stimulated by interleukin-1 β is believed to excite the chondrocytes in OA, causing an increased production of NO and PGE2 via the activation of NF- κ B [18–21]. To date, OA is not curable and no effective therapy exists which can prevent, stop, or retard OA progression. At present, many long-term therapeutic agents or drugs (e.g., nonsteroidal anti-inflammatory drugs) are available on the market to relieve pain and inflammation in OA; however, they are known to be associated with severe adverse effects of cardiovascular disease and the gastrointestinal tract. Therefore, we aimed in this study to investigate a nutraceutical-based safe and beneficial treatment to prevent, stop, or retard OA progression.

A number of polyphenolic flavonoids have been shown to exert strong anti-inflammatory effects and increasing interest for development of safe and effective treatment for OA [22]. Kaempferol (3, 5, 7, 4'-tetrahydroxy flavone) is a common dietary element and an important bioflavonoid that occurs in vegetables and fruits [23]. Kaempferol and its various glycosides exert a variety of pharmacological effects, such as anti-inflammatory, anti-cancer, anti-microbial, anti-diabetic,

anti-allergic, analgesic, cardioprotective, and neuroprotective activities [23–25]. Kaempferol has been extensively used as traditional therapy for several inflammatory diseases. Various previous pharmacological studies have shown that kaempferol can reduce iNOS and Cox-2 [26–29]. Moreover, some studies have explored the possible mechanism of kaempferol to restrain NF- κ B activity [29–33].

At present, to the best of our knowledge, anti-inflammatory and anti-osteoarthritis effects of kaempferol on the rat chondrocytes model of OA has not been reported. Therefore, we explored whether kaempferol could inhibit interleukin-1 β -induced inflammation in rat OA chondrocytes. We also examined the molecular mechanism of the protective effect of kaempferol on rat OA chondrocytes stimulated with interleukin-1 β .

Material and Methods

Chemicals and biochemical reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), kaempferol, and Griess reagent were obtained from Sigma-Aldrich Chemicals Pvt. Ltd. (St. Louis, MO, USA). Specific antibodies I κ B α , p-I κ B α , p65, and p-p65, and iNOS and Cox-2 were obtained from Cell Signaling Technology Inc. (MA, USA). ELISA kit was arranged from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant rat interleukin-1 β was procured from PeproTech (NJ, USA). All chemicals used were reagent grade. The animal experiment protocol was duly approved by the Institutional Animal Ethical Committee of Xiangyang Hospital Affiliation to Hubei University of Medicine (Hubei, China).

Cell culture conditions

The experimental rat chondrocytes and growth medium were obtained from Tianjin WeikaiBioeng Ltd. (Tianjin, China). Rat chondrocytes were incubated in chondrocytes growth medium at 37°C with 5% atmospheric CO₂ and replaced by a serum-free culture media overnight. In the Western blot study, resultant chondrocytes were treated with interleukin-1 β (10 ng/mL) alone or with kaempferol (25, 50, and 100 μ M) and interleukin-1 β (10 ng/mL) for two hours. In another experiment, chondrocytes were exposed to interleukin-1 β (10 ng/mL) alone or with kaempferol (25, 50, and 100 μ M, plus 200 μ M in MTT assay) and interleukin-1 β (10 ng/mL) for 24 hours. Rat chondrocytes cultured without kaempferol and IL-1 β were used as controls.

Cell viability assessment

In this study, kaempferol cytotoxicity was examined by MTT assay. In brief, chondrocytes at a density of 5×10^3 cells/well were seeded in 96-well plates overnight. The cultured wells

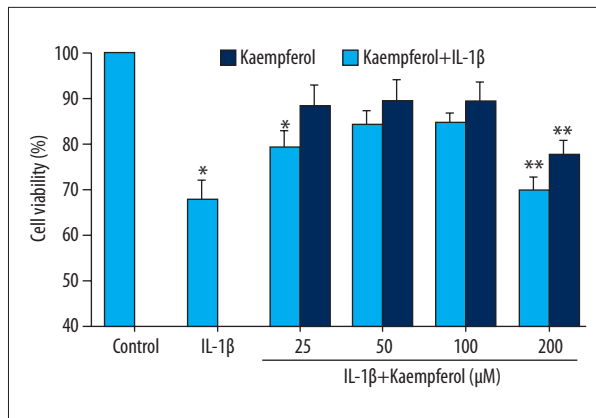


Figure 1. Effects of kaempferol on interleukin-1 β -stimulated cell viability in rat chondrocytes. The cytotoxicity effects of kaempferol were examined by MTT assay and cell cultures co-treated with different concentrations of kaempferol (25, 50, 100, and 200 μ M) and 10 ng/ml interleukin-1 β for 24 hours. Kaempferol (200 μ M) diminished the cell viability of chondrocytes. The data results indicated as mean \pm S E M. * p <0.05; ** p <0.01 compared to the control group (i.e., untreated with IL-1 β).

were stimulated with interleukin-1 β alone or with a various concentration of kaempferol (25, 50, 100, and 200 μ M) and interleukin-1 β for 24 hours. Then 20 μ L, 5 mg/mL MTT reagent was added and the plates were cultured for four hours at 37°C. The medium was separated and 100 μ L of dimethyl sulfoxide (DMSO) solvent was added to dissolve the insoluble formazan product. The absorbance was recorded at 490 nm using a micro-plate reader (Dynatech Labs, Chantilly, VA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-1 β -stimulated PGE₂ levels in chondrocytes were measured by an ELISA kit. In brief, chondrocytes were stimulated with interleukin-1 β (10 ng/mL) alone or with kaempferol (25, 50, and 100 μ M) and interleukin-1 β (10 ng/mL) for 24 hours. Finally the PGE₂ levels in the chondrocytes cultures were detected by an ELISA kit according to the instructions provided by the manufacturer.

Assessment of nitric oxide (NO)

In this experiment, interleukin-1 β -stimulated NO levels in chondrocytes were determined using Griess reagent (Sigma, G4410). In brief, chondrocytes were exposed to interleukin-1 β (10 ng/mL) alone or with kaempferol (25, 50, and 100 μ M) and interleukin-1 β (10 ng/mL) for 24 hours. Finally the NO levels in chondrocytes cultures were accessed using Griess reagent according to instructions provided by the manufacturer.

Western blot assessment

In the Western blot study, chondrocytes were treated with interleukin-1 β (10 ng/mL) alone or with kaempferol (25, 50, and 100 μ M) and interleukin-1 β (10 ng/mL) for two hours. The total proteins were extracted by Thermo Scientific M-PER[®] Mammalian Protein Extraction Reagent. In brief, proteins were estimated by bicinchoninic acid (BCA) assay. The samples of protein (adjusted with 20 μ g per lane) were dissociated by 10% SDS-PAGE under the reducing conditions. After the separation, the proteins were shifted to PVDF membranes and pre-incubated in blocking buffer (5% skimmed dried milk) for 30 minutes and subsequently incubated with specific antibodies I κ B α , NF- κ B, Cox-2, iNOS, p65 and p-I κ B α overnight at 4°C. The membranes were then washed with Tris-buffered saline with tween (TBST) and then incubated with secondary antibodies containing horseradish peroxidase (HRP) conjugated for two hours at 37°C. ECL detection reagent (Thermo) was applied to detect the membrane signal. The Quantity One software package (Bio-Rad) was applied to quantify the bands.

Statistical analysis

SPSS 13.0 software package was applied in the statistical study. The statistical data were indicated as the mean \pm SEM. All outcomes were determined using one-way ANOVA. Statistical significant difference is noted as p value <0.05.

Results

Effects of kaempferol on cell viability

The cytotoxicity of kaempferol on chondrocytes was evaluated at different concentrations (25, 50, 100, and 200 μ M). The results showed that interleukin-1 β -stimulated cell viability was significantly reduced compared to the untreated control at 24 hours. Conversely, kaempferol (up to 100 μ M) reversed the stimulation of interleukin-1 β and thus it did not show cytotoxic effects on chondrocytes (Figure 1). Therefore, we selected kaempferol (up to 100 μ M) in the subsequent assessment.

Kaempferol attenuated the PGE₂ and NO production

The anti-inflammatory properties of kaempferol were investigated by the estimation of PGE₂ and NO production in rat chondrocytes. Interleukin-1 β stimulation caused a significant elevated level of PGE₂ and NO. Nevertheless, it was observed that the levels of PGE₂ and NO concentration-dependently reduced in kaempferol treated rat OA chondrocytes stimulated with interleukin-1 β (Figure 2). Thus, kaempferol exhibited strong anti-inflammatory effects on interleukin-1 β stimulated rat OA chondrocytes.

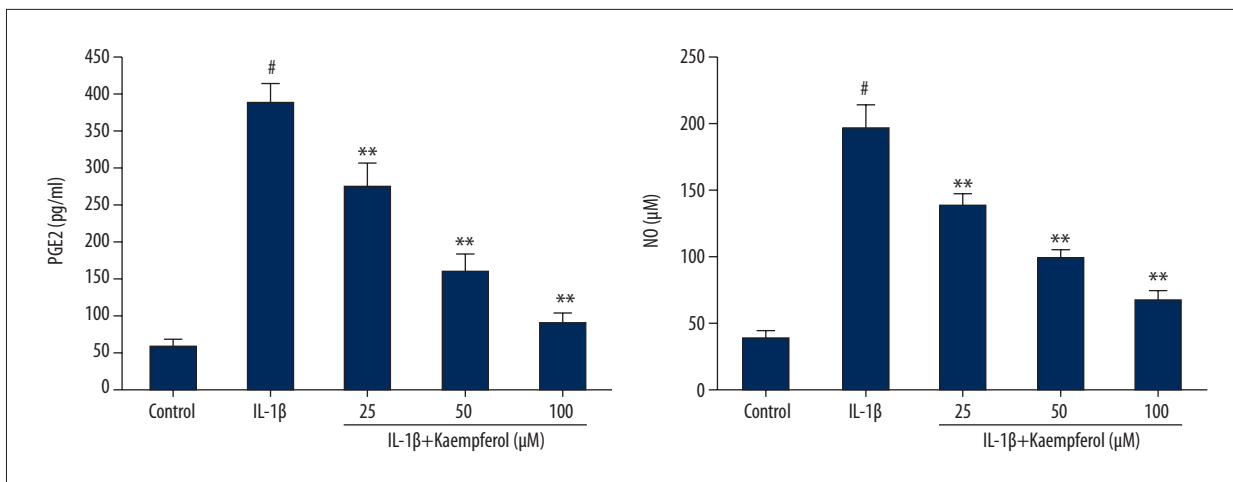


Figure 2. Kaempferol suppressed PGE₂ and NO production in IL-1β-stimulated rat chondrocytes. Anti-inflammatory properties of kaempferol (25, 50, and 100 µM) were determined by the estimation of PGE₂ and NO production. Rat chondrocytes were co-treated with different concentrations of kaempferol (25, 50, and 100 µM) and 10 ng/mL interleukin-1β for 24 hours. The data results are indicated as mean ± S E M. # *p*<0.05 compared to the control group (i.e., untreated with IL-1β); * *p*<0.05; ** *p*<0.01 compared to the IL-1β group.

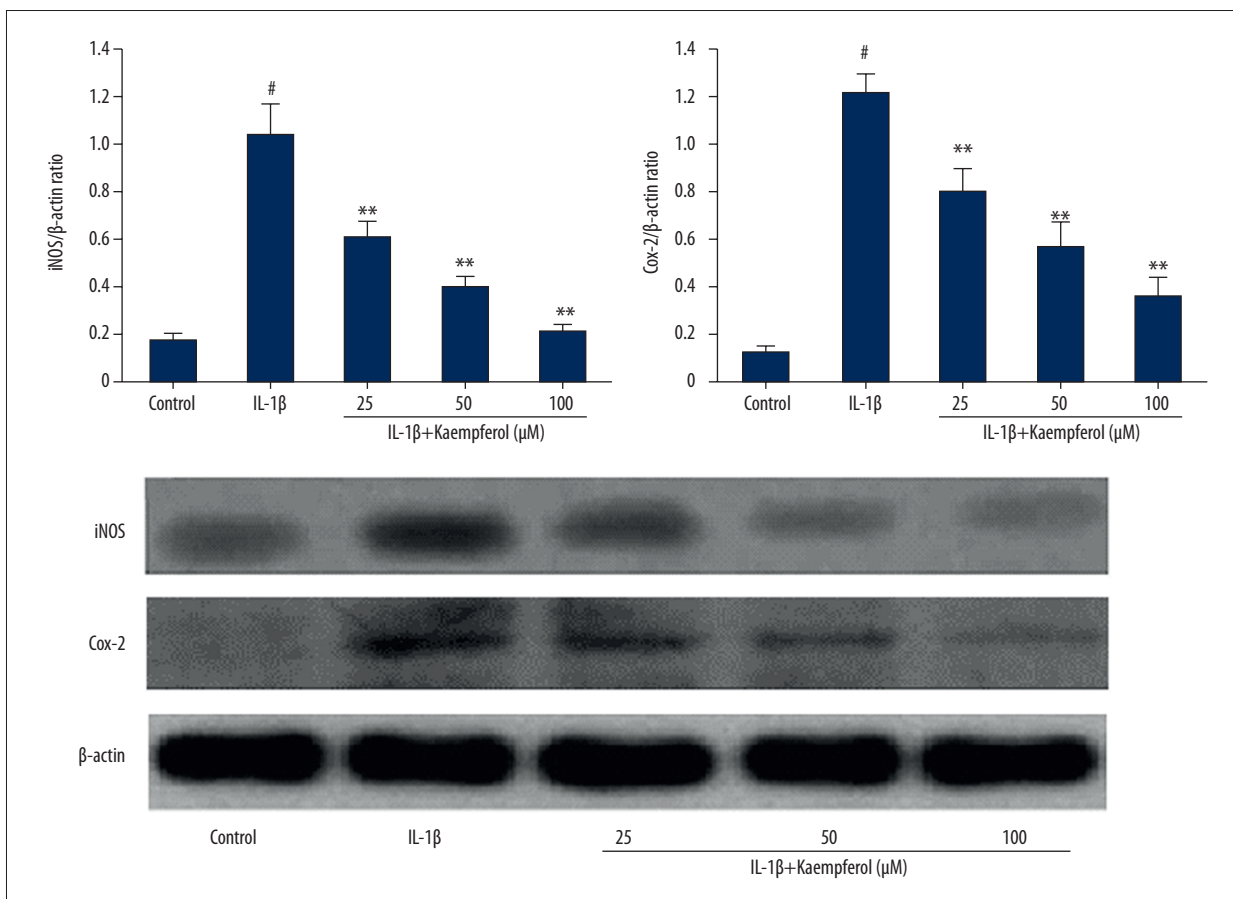


Figure 3. Kaempferol alleviated the iNOS and Cox-2 activity in interleukin-1β-stimulated rat chondrocytes. Western blotting was employed to determine the activity of iNOS and COX-2. Rat chondrocytes were co-treated with different concentrations of kaempferol (25, 50, and 100 µM) and 10 ng/mL interleukin-1β for two hours. The data results indicated as mean ± SEM. # *p*<0.05 compared to the control group (i.e., untreated with IL-1β); * *p*<0.05; ** *p*<0.01 compared to the IL-1β group.

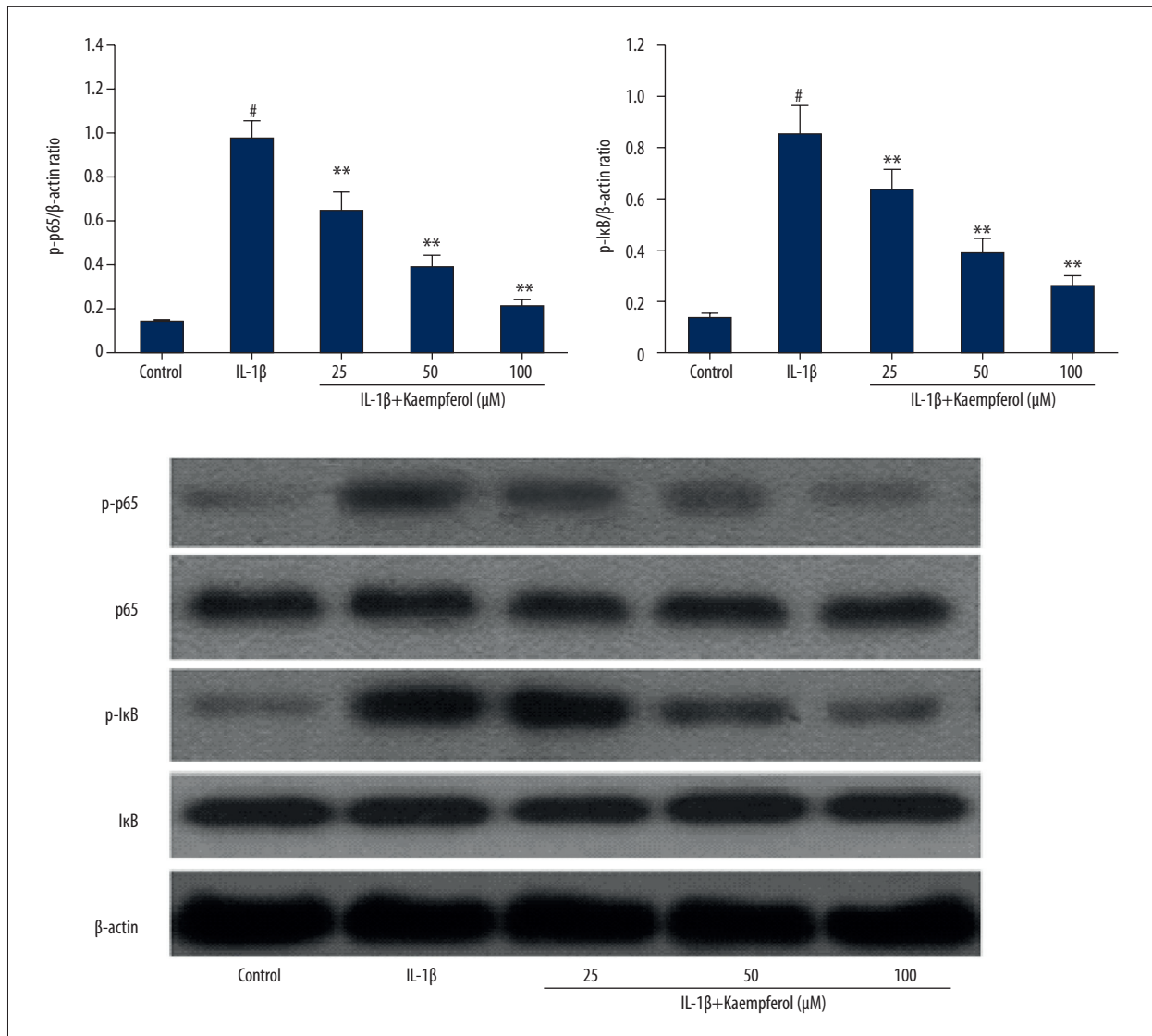


Figure 4. Kaempferol upturned IL-1 β -stimulated phosphorylation of I κ B α and NF- κ B p65 in rat chondrocytes. Rat chondrocytes were co-treated with different concentrations of kaempferol (25, 50, and 100 μ M) and 10 ng/mL interleukin-1 β for two hours. The data results indicated as mean \pm SEM. # $p < 0.05$ compared to the control group (untreated with IL-1 β); * $p < 0.05$; ** $p < 0.01$ compared to the IL-1 β group.

Kaempferol suppressed iNOS and Cox-2 protein expression

Our results showed that the protein expression of iNOS and Cox-2 were significantly raised in interleukin-1 β -stimulated chondrocytes. However, kaempferol (25, 50, and 100 μ M) treatment concentration-dependently alleviated inflammatory iNOS and Cox-2 activities (Figure 3).

Kaempferol inhibited inflammatory responses via NF- κ B pathway

The Western blotting showed that interleukin-1 β markedly stimulated the phosphorylation of I κ B α and NF- κ Bp65; however,

the phosphorylation was concentration-dependently upturned in kaempferol treated rat OA chondrocytes stimulated with interleukin-1 β . Therefore, kaempferol treatment notably attenuated the I κ B α degradation as well NF- κ B activation in the rat OA chondrocytes (Figure 4).

Discussion

Osteoarthritis (OA) is an age-related illness which leads to physical disability and reduces healthy life style. Presently, no effective treatment exists for OA. Nevertheless, many medicinal products are available to ease pain and inflammation in

OA, but most cause severe side effects to the cardiovascular system and the gastrointestinal tract. Thus, there is a strong need to develop a safe and effective natural compound for long-term treatment of OA. The present study explored a novel and therapeutically active agent naturally derived from dietary food which may prevent, stop, or retard the progression of OA.

Several reports have found that polyphenolic flavonoids exhibit strong anti-inflammatory activity, which is of immense interest for the development of safe and effective treatment for the OA [22]. Kaempferol is a polyphenolic flavonoid which richly occurs in various edible dietary plants. Several studies have shown that kaempferol and its various glycosides contribute pharmacological activities, including anti-inflammatory, anti-cancer, anti-microbial, anti-diabetic, anti-allergic, analgesic, cardioprotective, and neuroprotective activities [23–25]. To the best of our knowledge, anti-inflammatory and anti-osteoarthritis effects of kaempferol on rat OA chondrocytes stimulated with interleukin-1 β has not reported. Therefore, the present work investigated the anti-osteoarthritis effects of kaempferol and its associated anti-inflammatory mechanism on interleukin-1 β -induced in rat OA chondrocytes.

In this study, we demonstrated that kaempferol appeared to have a crucial protective and anti-inflammatory effect, possibly through the NF- κ B pathway in rat chondrocytes stimulated with IL-1 β . Supporting evidence from various research included findings that IL-1 β appears to have a significant role in the destruction of cartilage in OA and thus the inhibition of IL-1 β stimulated inflammatory mediators has been seen as a major aim in the OA treatment [12,17,34]. The excessive production of inflammatory cytokines, NO and PGE2 is commonly seen in the pathogenesis of OA [13,14,35]. Moreover, it has been widely seen that interleukin-1 β -stimulated iNOS and Cox-2 levels can lead to higher elevations of NO and PGE2. The inflammatory mediator NO is generally formed by the nitric oxide synthase (NOS) enzymes. Generally, cytokines stimulated inducible NOS (iNOS) enzyme triggers the production of NO. In our experimental analysis, we observed that kaempferol notably reduced NO and PGE2 levels (Figure 2) in addition to iNOS and Cox-2 activities (Figure 3) in interleukin-1 β -stimulated rat chondrocytes. This result strongly suggested that reduced production of PGE2 and NO by kaempferol treatment may be associated with the suppression of iNOS and Cox-2 activities.

These outcomes are similar to a study by Park et al. which found inhibitory effects of kaempferol on TNF- α , NO, PGE2, IL-1 β , and ROS formations as well as iNOS, Cox-2 activities in BV-2 microglial cells induced with the LPS [27]. In addition,

Kim et al. confirmed the potent anti-inflammatory effect of kaempferol to attenuate formation of NO, and PGE2 in addition to iNOS, and Cox-2 activities in the LPS stimulated macrophages [26]. Various published reports also suggest that kaempferol inhibits iNOS and Cox-2 [26–29]. This accumulation of evidence, together with our results, suggests kaempferol as a crucial anti-inflammatory dietary agent in the management of OA treatment. It is well-known and accepted that the induction of inflammatory mediators such as prostaglandin E2, cyclooxygenase-2 and nitrite, TNF- α and interleukin-1 β , are controlled by the NF- κ B pathway [36,37]. Therefore, a Western blot study was performed to investigate the possible mechanism of action, and whether kaempferol inhibits interleukin-1 β -stimulated NF- κ B signaling pathway in rat OA chondrocytes. In our work, we investigated the effects of kaempferol on NF- κ B because of its major involvement in the inflammatory process. Normally, NF- κ B is associated with I κ B α and is retained in the cytoplasm inactively. Once interleukin-1 β stimulates NF- κ B, it triggers the phosphorylation-mediated I κ B α degradation. Consequently, NF- κ B p65 translocates from the cytoplasm to the nucleus where it activates gene transcription, such as Cox-2 and iNOS [18,38,39].

The Western blot finding showed that kaempferol significantly inhibited the phosphorylation of I κ B α and NF- κ Bp65 (Figure 4). It can thus be postulated that kaempferol was preventing the phosphorylation-mediated I κ B α degradation as well as the nuclear translocation of NF- κ Bp65. Our results confirmed previous reported studies that demonstrated a strong anti-inflammatory effect of kaempferol through the NF- κ B signaling pathway [29–33]. The present investigation findings suggest that the protective effects of kaempferol on the experimental rat chondrocytes model of OA was by suppressing

PGE2 and NO formations, and iNOS and Cox-2 activities. Furthermore, kaempferol has also been found to restrain the activation of the NF- κ B pathway. However, further studies and clinical assessment are needed to validate this effect.

Conclusions

Kaempferol significantly suppressed IL-1 β stimulated inflammation responses in rat OA chondrocytes by inhibiting the NF- κ B pathway. Kaempferol exhibited strong anti-inflammatory and anti-arthritis effects in this study. Therefore, kaempferol is proposed to have potential benefit in prevention of the progression of OA.

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