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Research Article
Effects of Diosmin on IL-1β- Induced Inflammatory Response in Primary Rat Chondrocytes

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Abstract

Background and Objective: Diosmin, a bio-flavonoid present in the dietary supplement in citrus fruits. Due to its notable anti-inflammatory properties with less toxic effects, the potential chondroprotective activity was investigated. The aim was to investigate the chondroprotective activity of diosmin on IL-1β-stimulated inflammatory reaction in Primary Rat Chondrocytes (PRC) using an *in-vitro* model. **Materials and Methods:** PRCs were isolated and cultured followed by stimulation with IL-1β. The viability of cells was determined using an MTT assay. Nitric Oxide (NO) synthesis was estimated using the Griess Reaction (GR) method and the concentrations of PGE_2, MMP1, MMP13, TNF-α, IL-6 and COX-2 secreted by chondrocytes was assessed using the sandwich ELISA technique. **Results:** It was found that 50 and 100 μg mL^{-1} of diosmin had no effect on the viability of chondrocytes and were chosen for the study. The diosmin pre-treatment suppressed the induction of NO and PGE_2 in a dose-dependent manner. **Conclusion:** The results indicated that diosmin dose-dependently suppressed the inflammatory response by significantly reducing the production of NO, PGE_2, MMP1 and MMP13 in IL-1β challenged PRCs. Moreover, diosmin was able to cause the decrease in the levels of TNF-α, IL-6 and COX-2 in PRCs, thereby exhibiting the chondroprotective activity.

Key words: Diosmin, primary rat chondrocytes, anti-inflammatory, chondroprotective activity, IL-1β

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chondrocytes are the sole cellular component of articular cartilage and are responsible for the synthesis of the large turnover volume of Extracellular Matrix (ECM) components and maintenance of tissue homeostasis. Impairment in the chondrocyte function is linked with the development of degenerative diseases such as Osteoarthritis (OA)[1]. OA is the most prevalent musculoskeletal disease that affected nearly 100 M people globally having age over 45 years, which is about 15% of all cartilage disorders. Europe and the USA reflect the highest worldwide frequency of OA. Osteoarthritis grade in India shows nearly 80% of OA affected population[2,3]. The OA is a Rheumatic Musculoskeletal Disorder involving progressive cartilage degeneration, synovial inflammation, osteophyte formation, diminishing of joint space and subchondral sclerosis[4].

In response to stimulation by interleukin-1β (IL-1β), chondrocyte hypertrophy occurs which causes expression of hypertrophic markers such as runt-related transcription factor 2 (Runx2), ColX and matrix metalloproteinases-13 (MMP13) along with the increased release of neoepitopes, inflammatory mediators and increased innervation with associated release of neuropeptides and sensitizing agents[5]. This pathological stimulation leads to proteolytic enzyme expression and collagen network breakdown causing apoptosis of articular chondrocytes and disrupting cartilage integrity. Such changes are correlated with the release of nociceptive sensitizers of inflammatory reactions such as cytokines and neuropeptides[6]. Accumulating evidence suggests that sequential signalling cascades and the expression of proteolytic enzymes like MMP1 and MMP13 serve as primary factors that lead to the overall degradation of the collagenous framework[1]. Inflammatory cytokines induced by interleukin IL-1β causes upregulation of markers such as inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), IL-6, nitric oxide (NO) and prostaglandin E_2 (PGE_2). Previous research also reveals that IL-1β increases NO production enhances cyclooxygenase 2 (COX-2) activity and upregulates the expression of MMPs, including MMP13, which is linked with the degradation of Extracellular Matrix (ECM) and cartilage degeneration[7,8]. Therefore, an integrated study about potential biomarkers can provide evident data for the identification and advancement of therapeutically active drugs to attenuate the progression of OA.

Diosmin (diosmetin 7-O-rutinoside), a flavone glycoside of diosmetin, is obtained from citrus fruit peels as a dietary supplement, which is known for its potent anti-inflammatory and free radical scavenging properties. Flavonoids, including diosmin are broadly employed in biochemistry and pharmacology for their *in vivo* and *in vitro* anti-inflammatory, immunomodulatory and antioxidant effects[9,10]. Although diosmin exhibits its potential antioxidant effects, by scavenging reactive oxygen species and anti-inflammatory activity, Hence, the present study was aimed to demonstrate *in vitro* anti-osteoarthritic, the chondroprotective potential of diosmin on IL-1β-induced inflammatory response in primary rat chondrocytes.

MATERIALS AND METHODS

Study area: This study was carried out in May, 2020-2021.

Drugs and chemicals: All analytical grade reagents were used in the following research. Diosmin, type IV collagenase, IL1-β and kits for NO, PGE_2, MMP1, MMP13, TNF-α, IL-6 and COX-2 were obtained from Sigma-Aldrich, USA. Pronase was acquired from Roche, Basel, Switzerland. Dulbecco's Modified Eagle's Medium and fetal bovine serum was procured from Gibco Inc., NY, USA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Thermo Fisher Scientific, USA. Cytotoxicity assay and valuation of inflammatory markers were done on a microplate reader (Thermo Fisher Scientific, USA).

Chondrocytes isolation and culture: Primary Rat Chondrocytes (PRCs) were isolated from the knee joints of 4-weeks-old Sprague-Dawley rats and the tissues were enzymatically digested with 10 g L^{-1} of pronase and 1 g L^{-1} of type IV collagenase for 30 min and 6 hrs respectively at 37°C[11]. The Single-Cell Suspension (SCS) of chondrocytes was prepared by removing undissociated cells and debris through filtration. The obtained SCS was then centrifuged at 3600 rpm for 10 min. Chondrocytes were resuspended after centrifugation in Dulbecco's Modified Medium Eagle (DMEM). Cells were seeded in monolayer cultures formed at a concentration of 6×10^6 cells mL^{-1} in an antibiotic medium containing penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}) along with 10% Fetal Bovine Serum (FBS) in culture flasks and incubated at 37°C in a humidified incubator comprising 5% CO_2. The culture medium was replaced every 3 days[12]. To investigate the chondroprotective effect of diosmin, the PRCs were pre-treated with 0.1% dimethyl sulfoxide (DMSO, control) or various concentrations of diosmin for 1 hr and stimulated with IL-1β (10 ng mL^{-1}) for 24 hrs[13].

Cell viability assay: The isolated chondrocytes were seeded into a microplate of 96 well sizes at a density of 5×10^3 cells per well. After pre-treatment with diosmin and IL-1β stimulation,

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay was performed to assess cell viability and to evaluate the cytotoxic effects of diosmin. This was done conferring to the manufacturer's protocol. In each well, 100 µL of MTT were added and incubated for 4 hrs[12]. To dissolve the crystals, supernatants were supplemented with 150 µL DMSO and agitated for 10 min, followed by measuring absorbance at 490 nm using a microplate reader. Percent viability was estimated using equation:

$$\text{Viability (survival) \%} = \frac{OD_{test}}{OD_{control}}$$

where, OD indicates optical density[14]

NO assay: NO production was evaluated spectrophotometrically by using Griess Reagent (GR) to estimate the nitrite concentrations in the supernatant cell culture according to the manufacturer's instructions. Chondrocytes were pre-treated with diosmin for 2 hrs and then stimulated with IL-1β (10 ηg mL^{-1}). After 24 hrs, the supernatants were obtained and each supernatant was added to an equal volume of GR (150 µL) and incubated at room temperature for 5 min. Absorbance was then measured at 540 nm[14].

ELISA: Cell supernatant of the chondrocytes pre-treated with diosmin and stimulated with IL-β (10 ng mL^{-1}) was collected and the levels of PGE_2, MMP1, MMP13, TNF-α, IL-6 and COX-2 secreted by chondrocytes were confirmed by sandwich ELISAs and all the assays were carried out as per the manufacturer's protocol.

Statistical analysis: Results were expressed Mean±SEM and the analysis was done using analysis of one-way variance (ANOVA), followed by Tukey's Multiple Comparison Test using GraphPad Prism version 8.1. P. Differences were deemed as statistically significant when $p<0.05$.

RESULTS

Effect of diosmin on IL1β stimulated PRCs cell viability: MTT assay performed to determine the effects of diosmin at various concentrations viz., 50, 100, 200, 300, 600, 1000 and 2000 µg mL^{-1} on the viability of IL-1β-induced inflammatory response in PRCs demonstrated as 99.98±0.12, 99.92±0.14, 97.63±0.20, 94.44±0.15, 78.22±0.09, 50.30±0.25 and 23.98±0.11, respectively in Table 1. It was found that 50 and 100 µg mL^{-1} of diosmin did not affect the viability of chondrocytes. Therefore, 50 and 100 µg mL^{-1} were chosen for further studies.

The results indicate that IL-1β significantly induced the expression of NO and PGE_2 compared to that in the control group. However, diosmin pre-treatment suppressed the induction of NO and PGE_2 in a dose-dependent manner as follow, NO -99.72±1.76 (control), 57.42±1.02 (diosmin 50 µg mL^{-1}) and 35.40±0.94 (diosmin 100 µg mL^{-1}) and PGE_2 -1054.42±11.82 (control), 763.28±1.13 (diosmin 50 µg mL^{-1}) and 459.44±0.82 (diosmin 100 µg mL^{-1}) in Table 2. The effect of diosmin on the levels MMP1 and MMP13 of IL-1β challenged PRCs was estimated using ELISA tests. The results displayed that the levels of MMP1 and MMP13 increased significantly after IL-1β treatment as follow, MMP1- 233.36±4.27 (control), 104.02±1.32 (diosmin 50 µg mL^{-1}) and 80.15±1.01 (diosmin 100 µg mL^{-1}) and MMP13 -206.74±3.51 (control), 98.24±1.70 (diosmin 50 µg mL^{-1}) and 75.37±1.21 (diosmin 100 µg mL^{-1}). However, diosmin pre-treatment inhibited IL-1β induced MMP1 and MMP13 levels in a dose-dependent manner.

Further, to verify the inhibitory effect of diosmin on IL-1β-induced PRCs, the expression levels of TNF-α and IL-6 and COX-2 were evaluated using ELISA kits. PRCs were pre-treated with various concentrations of diosmin for 2 hrs before subsequent IL-1β stimulation. The results confirmed that pre-treatment with diosmin significantly suppressed IL-1β-mediated increase in the expression of TNF-α, IL-6 and COX-2 levels as follow, TNF-α -152.21±1.94, 91.64±1.66 and

Table 1: Outcome of diosmin on the viability of PRCs in presence of IL-1β

Diosmin (µg mL^{-1})	Percentage viability of PRCs (Mean±SEM) (n = 3)
50	99.98±0.12
100	99.92±0.14
200	97.63±0.20
300	94.44±0.15
600	78.22±0.09
1000	50.30±0.25
2000	23.98±0.11

Data are expressed as Mean±SEM (n = 3)

Table 2: Effect of diosmin on NO, PGE_2, MMP1 and MMP13 levels in PRCs

Treatments	NO (µmol)	PGE_2 (pg mL^{-1})	MMP1 (ηg mL^{-1})	MMP13 (ηg mL^{-1})
Control	99.72±1.76	1054.42±11.82	233.36±4.27	206.74±3.51
Diosmin (50 µg mL^{-1})	57.42±1.02**	763.28±1.13**	104.02±1.32***	98.24±1.70***
Diosmin (100 µg mL^{-1})	35.40±0.94***	459.44±0.82***	80.15±1.01***	75.37±1.21***

Data are expressed as Mean±SEM (n = 6), **p<0.01 and ***p<0.01 considered statistically significant as compared to control

Table 3: Effect of diosmin on TNF-α, IL-6 and COX-2 levels in PRCs

Treatments	TNF-α (pg mL^{-1})	IL-6 (pg mL^{-1})	COX-2 (ηg mL^{-1})
Control	152.21±1.94	135.28±1.04	21.48±0.80
Diosmin (50 μg mL^{-1})	91.64±1.66**	91.64±0.88**	15.62±0.28**
Diosmin (100 μg mL^{-1})	75.92±1.24***	75.92±1.12***	10.06±0.48***

Data are expressed as Mean±SEM (n = 6), **p<0.01 and ***p<0.01 considered statistically significant as compared to control

75.92±1.24, IL-6 -135.28±1.04, 91.64±0.88 and 75.92±1.12 and COX-2 -21.48±0.80, 15.62±0.28 and 10.06±0.48 of three groups viz., control, diosmin 50 μg mL^{-1} and diosmin 100 μg mL^{-1}, respectively Table 3.

DISCUSSION

IL-1β is considered one of the key players involved in the destruction of PRCs aggravating the progression of OA. Chondrocytes are cellular cartilage components responsible for maintaining ECM and balancing anabolic and catabolic metabolism under normal conditions[15]. IL-1β autonomously and in tandem with certain mediators causes inflammatory reactions and catabolic responses concerning the articular cartilage and other joint components[16]. Frequently-used pharmacological treatments, such as oral non-steroidal anti-inflammatory drugs (NSAIDs), for the management of patients with OA are often associated with serious adverse effects[17]. Consequently, plant-derived agents with anti-inflammatory activity and low toxicity are more widely used as a therapeutic option for reducing such inflammatory responses[18]. Diosmin is a bioactive flavonoid isolated from plants and demonstrates anti-inflammatory properties in several studies[7,19]. There is no report of diosmin on inflammatory response in PRCs was found. In this context, an attempt was taken to investigate the anti-inflammatory and cartilage-protective effects of diosmin on IL-1β-stimulated rat primary chondrocytes in the present study.

Inflammatory cytokines have a predominantly detrimental impact on articular cartilage and several studies have also reported that the action of IL-1β blocks chondrocytes in the context of ECM component synthesis and interferes with the synthesis of main structural proteins, such as Type-II collagen and aggrecan[20]. In addition, the IL-1β also affects the functioning of chondrocytes in the synthesis of enzymes from the metalloproteinase group (MMPs), especially interstitial collagenase (MMP1), stromelysin-1 (MMP3) and collagenase 3 (MMP13), which have a crucial impact on cartilage components[21,22]. Thus, the agent that inhibits the development of inflammatory cytokines and MMPs may serve as a new therapeutic agent for OA. The present research revealed that treatment with diosmin reduced the increased levels of MMP1 and MMP13.

IL-1β can induce its secretion in the joint cells in an autocrine manner to stimulate the synthesis of other cytokines such as TNF-α and IL-6. Both of them induce the production of iNOS, COX-2 and PGE_2 synthase, thereby increasing the amounts of their products[23]. The PGE_2 is produced from arachidonic acid through the stimulation of COX-2 during an inflammatory response. The NO is obtained from the amino acid L-arginine as a consequence of the enzymatic action of iNOS, which is induced by inflammatory stimuli, indicating the upregulation of proinflammatory genes, iNOS and COX-2 in chondrocytes, which are responsible for the beginning of IL-1β stimulated PRCs destruction[12,20]. Therefore, the effect of diosmin on the secretion of NO and PGE_2 from PRCs in the presence of IL-1β was investigated. It was found out that inflammatory intermediaries such as NO and PGE_2 were significantly reduced by diosmin in PRCs, which probably was due to a decrease in iNOS and COX-2 expressions. Besides this, elevated levels of IL-6 and TNF-α were also suppressed by diosmin treatment. IL-6 is considered as the major cytokine triggering impairment in the subchondral bone layer[23,24]. This effect is based largely on promoting the formation of osteoclasts and therefore bones resorption while synergizing with IL-1β and TNF-α[24]. On the other hand, diosmin also reduced the elevated COX-2 level. Thus, the suppressing of TNF-α, IL-6 and COX-2 by diosmin can be attributed to the chondroprotective activity of the bioflavonoid diosmin.

In an *in vitro* anti-inflammatory study, diosmin inhibited acetic acid-induced inflammatory reaction in rat colon through the clampdown of inflammatory and oxidative stress markers[25]. Therefore, in the present study decrease in levels of TNF-α and IL-6 is possibly due to the downregulation of proinflammatory genes in diosmin treated PRCs.

CONCLUSION

Collectively, pre-treatment with diosmin effectively inhibited IL-1β stimulated inflammatory factors (NO, PGE_2, TNF-α, IL-6 and COX-2). Moreover, diosmin also suppresses cartilage-degrading enzymes (MMP1 and MMP13) and

protects constitutive components of chondrocyte ECM from degradation by IL-1β treatment. The above findings suggest that diosmin can play a potential chondroprotective role in IL-1β-induced inflammatory response. Besides, studies should provide a vivid picture of the molecular mechanisms underlying the effects of diosmin on chondrocytes, which could make it a potential candidate for the progress of new therapeutic drugs shortly. Furthermore, *in vivo* research of diosmin in both animals as well as a human is essential to further investigate the potential anti-inflammatory action of diosmin on chondrocytes.

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This study discovered the effect of diosmin on inflammatory response induced by IL-1β in PRC that can be beneficial for inflammatory research. This study will help the researchers to uncover the critical areas of inflammation that many researchers were not able to explore.

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