

Evaluation of Transforming Growth Factor Beta 1 and Curcumin on Proliferation and Differentiation of Nasal-Derived Chondrocyte Seeded on the Fibrin Glue Scaffold

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ABSTRACT

Introduction: Natural biomaterials and growth factors are key factors in tissue engineering. The objective of the present study was to evaluate transforming growth factor beta 1 (TGF- β 1) and curcumin on proliferation and differentiation of nasal-derived chondrocyte seeded on the fibrin glue scaffold.

Methods: Chondrocytes were isolated from nasal samples. Nasal-derived chondrocytes were seeded on fibrin glue at chondrogenic induction medium for 2 weeks. In this study, the effects of various concentrations of curcumin and TGF- β 1 on the survival and proliferation of chondrocytes seeded on fibrin biomaterial were assessed by MTT assays. Also, chondrocyte-specific gene expression was assessed by real-time polymerase chain reaction (PCR).

Results: There were significant differences among the group treated with curcumin 10 μ g compared to other groups with regard to cell viability. Also, gene expression of collagen type II, aggrecan, and SOX9 in the chondrocytes seeded on fibrin biomaterial containing the growth factor TGF- β 1 significantly differed from those of curcumin and control group.

Conclusion: Our results indicate that TGF- β 1 and natural biomaterial of curcumin can be used effectively in chondrogenic viability and differentiation of nasal-derived chondrocyte.

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1. Introduction

Articular cartilage is known as a connective tissue with low cellular activity that limits its potential for regeneration and repair [1, 2]. Tissue engineering (TE) provides a technique to reconstruct the cartilage defects. Identification of the optimal condition for regeneration is one of the major factors in TE [3-6]. Articular cartilage cannot repair by itself. This shortcoming later leads to arthritis. Arthritis is the most common skeletomuscular disease of old age. In 2012, arthritis was reported as the fourth cause of disability [7-10]. There are several treatments such as chondrocyte transplantation and drilling for current disabilities. Although these treatments have advantages, they can produce fibrous tissue instead of hyaline cartilage [11].

Nowadays, cell therapy using tissue engineering has become a promising way for repairing injured articular cartilage, which contains chondrocyte biomaterials and appropriate cultural environment [12]. It is proven that transplantation of monolayer chondrocytes culture helps the chondrocytes look like fibroblasts. As regards cell choices, use of differentiated cells in TE is limited due to many factors. Despite the fundamental requirement of cell expansion to achieve a sufficient number of cells for TE, differentiated cells have limited proliferation potency in culture. Furthermore, differentiated cells often lose their tissue-forming capacity in vitro and may dedifferentiate in conventional monolayer culture and their phenotype changes in vitro. On the other hand, the researchers showed that maintenance of the chondrocyte phenotype could be achieved by using 3D structure, herbal extracts, and growth factors. These scaffolds provide voided structures for attachment and growth of cells and their suspension with high density and desirable stimulation at these 3D structures [13]. In the last decade, the capacity of natural scaffolds for support of cell growth has been tested. Fibrin obtained from blood has been used in clinical treatments. Several advantages of fibrin include excellent biocompatibility, promotion of the cell adhesion, and lack of immunogenicity [14].

Several studies have been done regarding the effects of some plants in treating osteoarthritis and joint disorders, among which turmeric has proven to be the most effective one [15-17]. Curcumin is the effective component of turmeric with chemical name of feruloylmethane. Curcumin has various water soluble components with anti-inflammation, apoptosis, anti-catabolic, and strong antioxidant effects. It has a high solubility in ethanol, dimethyl solf-

oxide and other organic solvents. Curcumin molecular weight is 368.37 and its melting point is 183°C [18, 19]. Commercial curcumin is made of diferuloylmethane (82%) and its derivatives demethoxycurcumin (15%) and bisdemethoxycurcumin (3%). Some of curcumin effects include loading to reduce tumor cell survival, expansion, and secondary inflammation through NF-KB inhibition, as well as suppression of constitutive phosphorylation IKBa via the presentation of IKB kinase [20-24]. In this study, we evaluated the comparative efficacy of TGF-β1 and curcumin on the proliferation and differentiation of nasal-derived chondrocyte seeded on the fibrin glue scaffold.

2. Materials and Methods

Materials

Dulbecco's minimum essential medium (DMEM) for cell culture was purchased from Sigma and fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (BRL, Gaithersburg, MD, USA).

Isolation of chondrocyte cells

The specimens were obtained by rhinoplasty after informed consents were obtained from patients. Next, the specimens were carried from the hospital to the laboratory (under sterilized condition: phosphate buffered saline containing 2% penicillin streptomycin). They were washed by phosphate buffer saline (PBS) 3 times, then divided into smaller pieces. After that, these pieces were digested by collagenase type II so that nasal-derived chondrocytes got isolated. They were incubated for 4-5 hours at 37°C and then centrifuged for 10 minutes at 1800 rpm. Then, the supernatant was eliminated and pellet was cultured in DMEM containing 1% penicillin, streptomycin, and 10% FBS at 95% humidity, 5% CO₂, and at 37°C. After 24 hours, the adherence cells have been removed and cells became ready to use at P3.

Expression analysis of chondrogenic genes by real time PCR

Expression of cartilage-specific genes (type I and II collagen, Sox9 and aggrecan) was evaluated after 14 days as previously described. All of the samples were digested within liquid nitrogen and then total RNA was extracted using an Accuzol™ (BioNEER) in accordance with the manufacturer's protocol. Afterwards, the reverse transcription of RNA was carried to produce a complementary DNA (cDNA) using the Accupower® RTpreMIX (BioNEER). Real-time PCR was performed using SYBR

Green PCR Master Mix and Rotor-Gene™ 6000 Series software version 1.7.65 (Corbett Life Science), real time-PCR performance using SYBR Green PCR Master Mix (Amplicon) and Rotor-Gene™ 6000 Series software version 1.7.65 (Corbett Life Science), and primers of each gene were designed as follows utilizing primer 3 program (Table 1).

The reaction was initiated by heating at 95°C for 15 minutes, followed by 40 cycles of elongation at 59°C for 30 seconds and denaturation at 95°C for 15 seconds. Target gene was normalized based on glyceraldehyde 3-phosphate dehydrogenase reference gene. The level of expression of each target gene was calculated using $2^{-\Delta\Delta CT}$.

Histological examination

Scaffolds from 2 groups containing TGF-β1 and curcumin were analyzed histologically. After fixation with 10% neutral buffered formalin for 24 hours, samples were embedded within paraffin and sectioned at 5 μm thickness. For histological evaluation, sections were deparaffinized, rehydrated through a series of graded ethanol, and stained with hematoxylin/eosin.

Statistical analysis

Significant differences in expression levels of 4 genes in construct were identified by the least significant difference (LSD) test for the real-time PCR analysis. This reaction was repeated 4 times for every sample. Data were analyzed statistically using SPSS software (version 17). Significance was considered at $P < 0.05$.

Cell proliferation and viability

Cell viability and proliferation were assessed by MTT at day 14. Our study identified that in 2 intervention groups, viability and proliferation of cells significantly increased

compared to the control group (cells without TGF-β1 and curcumin) ($P < 0.05$).

Preparation of fibrinogen and thrombin

Fibrinogen and fresh frozen plasma solutions (FFP) and thrombin were obtained from the Iranian Blood Transfusion Organization. FFP was thawed in water bath at 37°C. Then, 15 mL of it was mixed with 10 mL of calcium gluconate (mid 5:3 ratio). The solution was incubated for 1 hour at 37°C and subsequently centrifuged at 2200 rpm for 10 minutes. The supernatant was collected as thrombin. Fibrinogen and thrombin solutions were prepared for use as cell culture. To this end, the chondrocyte cells at a concentration of 1×10^6 cells per mL were dissolved within fibrinogen and then thrombin was added to them. The chondrogenic medium was prepared as follows: DMEM high glucose, including insulin-transferin-selenium 1%, dexamethasone 100 nM, bovine albumin serum 1%, 1.5 μg linoleic acid, 50 μg/mL ascorbic 2-phosphate and penicillin streptomycin 1%, and 10 ng/ml TGF-β1/10 μm curcumin.

3. Results

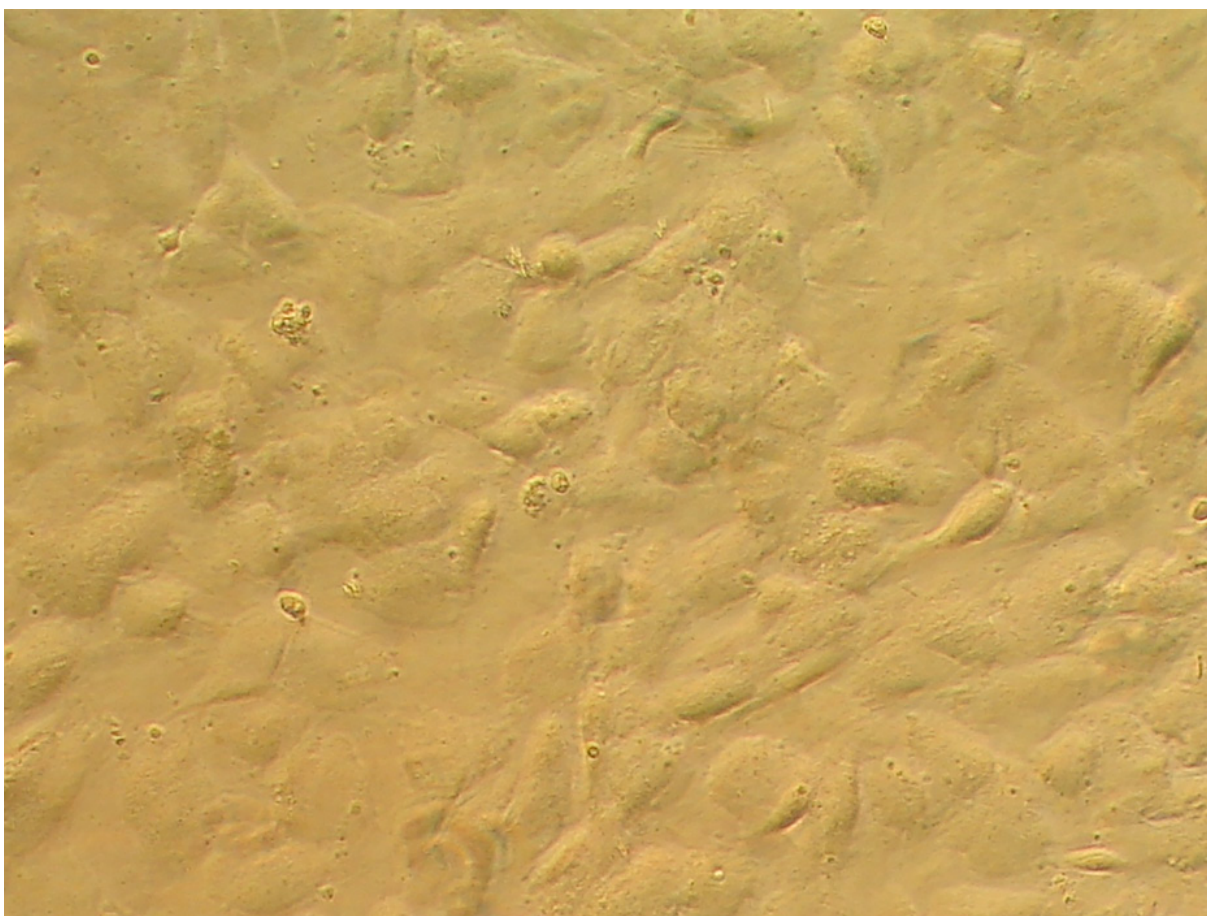
Chondrocyte characterization

Human chondrocytes were identified by spindle shape, fibroblast-like morphology in the primary culture (Figure 1). Homogeneous cell population was determined at passage 3 (Figure 1). The cell survival in the group treated with 10 μg TGF-β1 compared to the other groups showed no significant difference (Figure 2). Also, this study showed that a significant difference existed in cell viability among the group treated with curcumin 10 μg compared to other groups (Figure 3). Chondrocyte-specific gene expression was performed in different groups using real-time PCR. In this study, it was shown that gene expression of collagen type II and aggrecan and Sox9 in the chondrocytes seeded

Table 1. Primer sequences used for real-time PCR.

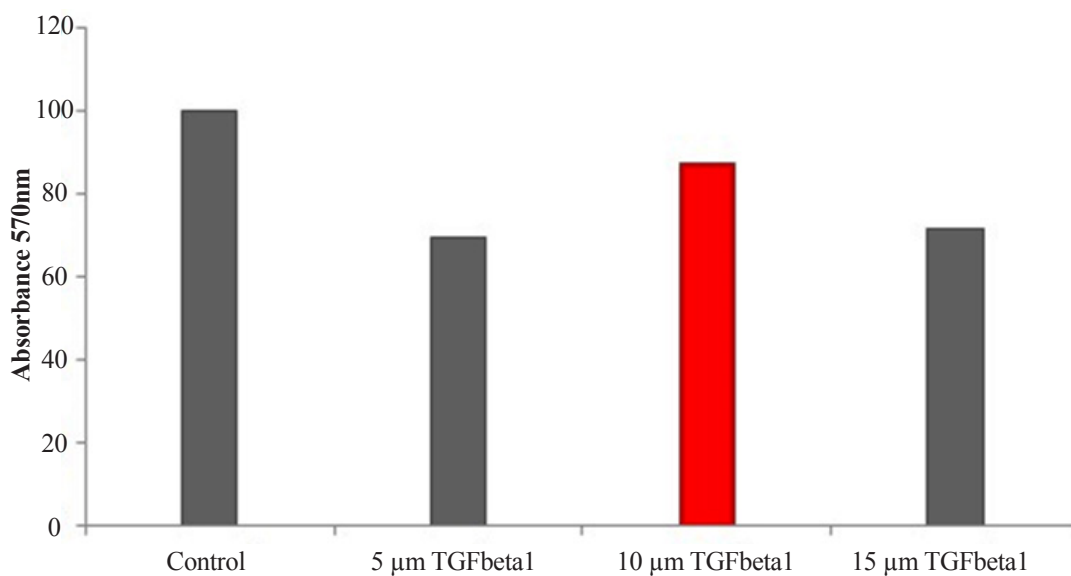
Primer name	Sequences (3'>5')	Reference
Collagen type I (COL2A1) forward Collagen type I (COL2A1) reverse	CGTCCAGATGACCTTCTACG TGAGCAGGCGCTTCTTGAG	NM_001844
Collagen type II (COL1A2) forward Collagen type II (COL1A2) reverse	CAGGAAACAGCTATGACC CTACTCTCAGCCAGGAGGTCTG	NM_000089
Aggrecan (ACAN) forward Aggrecan (ACAN) reverse	AGGCAGCGTGATCCTTACC GGCCTCTCCAGTCTCATTCTC	NM_001135
SOX9 Forward SOX9 Reverse	GTACCCGCACTTGCAAC TCTCGCTCTCGTTGAGAAGTC	NM_000346
GAPDH Forward GAPDH Reverse	CGCTCTGCTCCTCTGTT CCATGGTGTCTGAGCGATGT	NM_001256799

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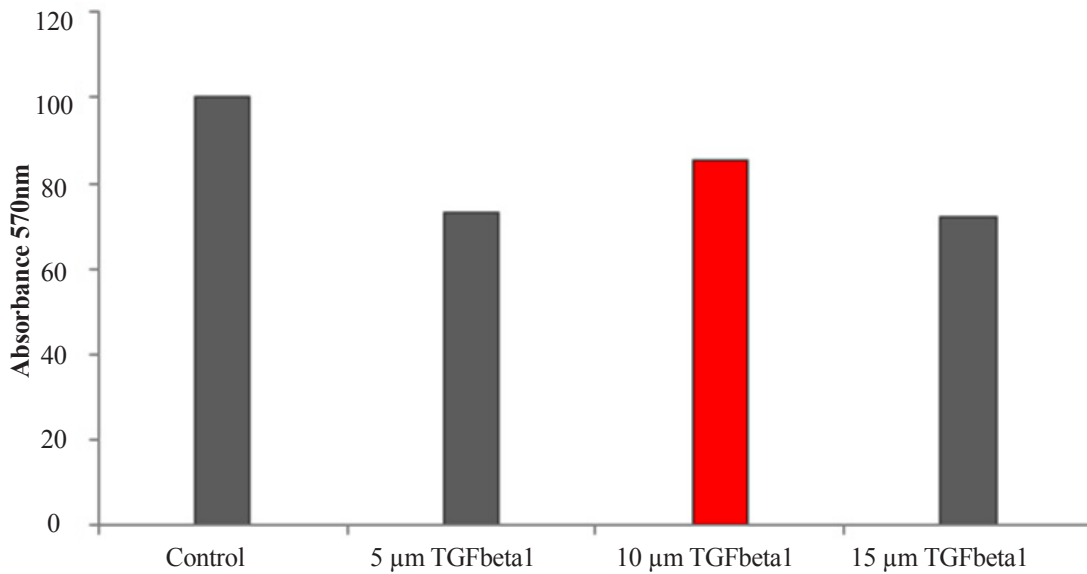
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Figure 1. Human nasal-derived chondrocytes of fibroblast-like observed by inverted phase-contrast microscope.



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Figure 2. MTT assay to evaluate the effects of different concentration of TGF-β1 on survival and proliferation of chondrocytes seeded on fibrin biomaterial.



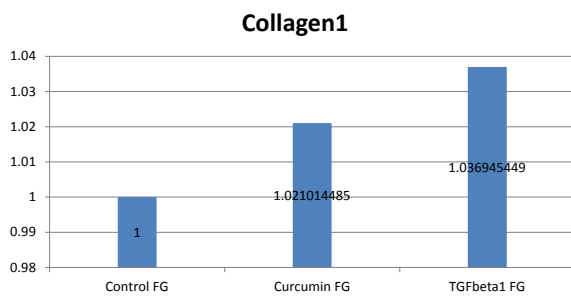
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Figure 3. MTT assay to evaluate the effects of different concentration of curcumin on survival and proliferation of chondrocytes seeded on fibrin biomaterial.

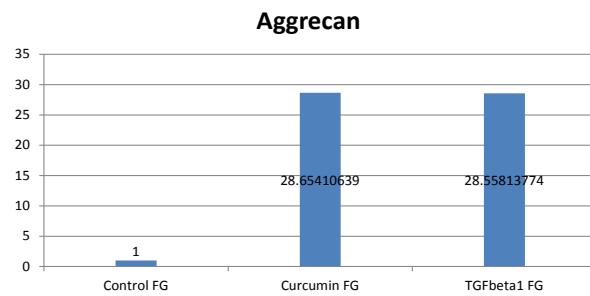
on fibrin biomaterial containing the growth factor TGF-β1 showed significant difference compared to curcumin and control groups (Figure 4).

In this study, the effect of various concentrations of curcumin and TGF-β1 on the survival and proliferation of chondrocytes seeded on fibrin biomaterial was assessed

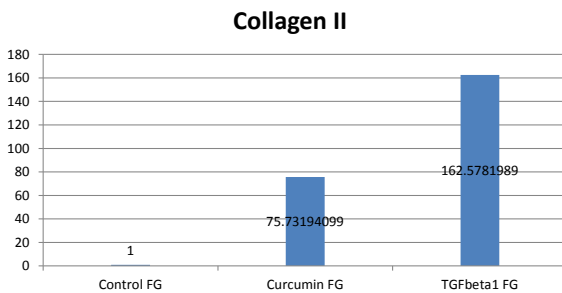
A



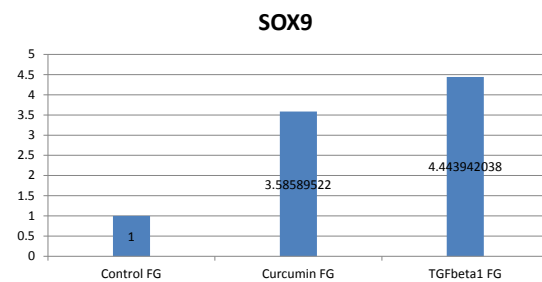
B



C

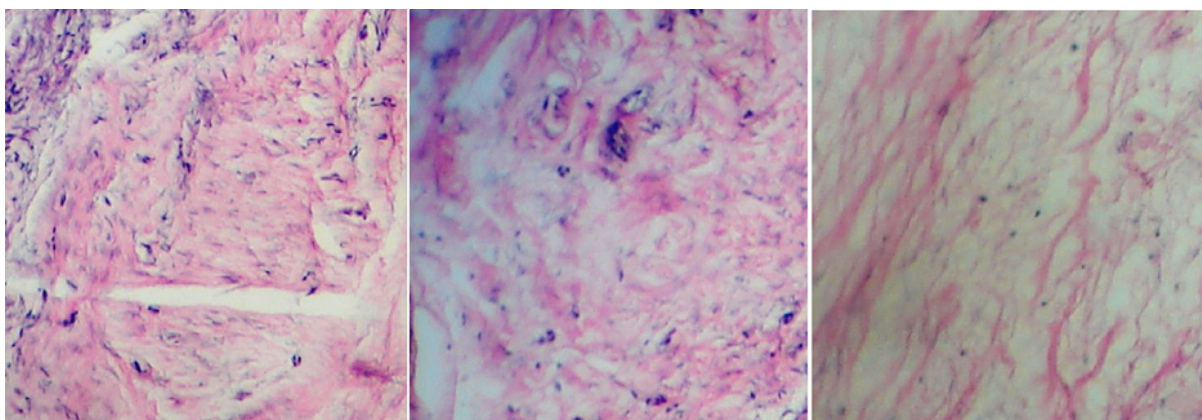


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Figure 4. The mRNA expression of SOX9, collagen Type II, Aggrecan and collagen Type I in the human chondrocytes seeded on fibrin biomaterial supplemented with TGF-β1, curcumin (A-D respectively). *Statically significant (P<0.05).



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Figure 5. Histological staining (40x) with H&E in the chondrocytes seeded on fibrin biomaterial containing the growth factor TGF- β 1 (A), in the chondrocytes seeded on fibrin biomaterial containing the growth factor curcumin (B), and the chondrocytes seeded on fibrin biomaterial as control (C).

by MTT assay. Chondrocytes were isolated from human nasal cavity. Histologically, chondrocytes seeded on fibrin glue contained matrix and many cells. There was histological evidence of more extensive cartilaginous matrix, also type II collagen content was significantly higher than in human chondrocyte/fibrin glue scaffold supplemented with TGF- β 1 (Figure 5).

4. Discussion

According to our study, there were differences between 2 intervention groups and the control group. Moreover, based on developmental biology, the availability of novel scaffold-cell systems *ex vivo* with tight environmental control can provide valuable information about tissue formation processes as well as new insight into how tissues regenerate. This would optimize the approaches towards tissue repair, which is currently dominated by uncontrolled inflammatory wound healing pathways (that leave scars).

Articular cartilage possesses hyaline cartilage with low metabolic rate due to lack of vascular supply. The major limitation of adult articular cartilage is the inability to repair. Even the most minor injuries may leave damages and osteoarthritis joint degeneration, causing severe pain and disability [25]. With regard to repairing cartilage defects, there are multiple techniques, including drilling, abrasion arthroplasty, mosaicplasty, as well as autologous and allogeneic chondrocyte transplantation. The main problem of autografts and allografts is possibility of disease transmission, immune response, limitation of remodeling and performance of many surgeries, and morbidity of harvesting sites. TE has shown a promising approach in the repair of cartilage tissue [22, 26-29].

Chondrocytes have the regenerative capability to synthesize cartilage-like matrix *in vitro* and protecting chondrogenic phenotype while cultured in 3D construct with suitable agents. A 3D structure provides an environment to facilitate chondrocytes adhesion, expansion, and proliferation as well as maintenance of the cellular phenotype and function [25, 30-33]. Zheng et al. showed that using matrix-induced autologous chondrocyte implantation can support chondrocyte differentiation and after 6 months about 75% of biopsies from patients confirmed hyaline-like cartilage formation [34].

TGF- β can stimulate the production of proteoglycans and other components of cartilage matrix in the various stages of the cartilage regeneration process [35]. Since a long time ago, it has been known that curcumin has the anti-inflammatory property. Curcumin (50 μ M concentration) has the ability to inhibit NF-KB activation. This agent can induce proinflammatory genes, Cox2, and VEGF [36].

Chowdhury et al. reported that curcumin (0.01-100 ng/mL) could inhibit activator protein (AP) -1 and consequently reverse the interleukin 1 beta stimulated production of nitric oxide and prostaglandin [37]. *In vitro* studies, human articular chondrocytes/alginate beads composite have been exposed to curcumin (1-20 μ M). This study showed nontoxic properties of curcumin on cell viability [38].

In another investigation, Mathy et al. showed that curcumin suppressed Cox2 gene expression compared to non-steroidal anti-inflammatory drugs [39]. Barman et al. used curcumin (5 μ M) and reported that it modulated inflammation in human tenocytes [40].

Huang et al. reported that curcumin was able to inhibit B-cell activating factor production in a mouse model [41]. In another study, Banji et al. showed that the combination of methotrexate and curcumin had anti-inflammatory effects and increased chondrocyte protective action [42]. The expression and secretion of various matrix metalloproteinase are regulated by the inflammatory and chondroprotective effects of curcumin [43-45]. In conclusion, curcumin can increase the expression of cartilage specific genes. Herbal extractions like curcumin have potential therapeutic effects on osteoarthritis.

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Conflict of Interest

The authors of this study declared no conflict of interests.

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