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Original Article

Transforming Growth Factor-β1 Preserves Bovine Nasal Cartilage against Degradation Induced by Interleukin-1α in Explant Culture

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ABSTRACT

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Background and Aims: Chondrocytes and their differentiation play a central role in joint diseases. Effect of the transforming growth factor (TGF)-β1 on chondrocyte characteristics and differentiation is not clearly understood. This study was undertaken to investigate the effects of TGF-β1 on tissue characteristics and morphology of chondrocytes against degradation induced by interleukin (IL)-lα in bovine nasal cartilage (BNC) explant culture.

Materials and Methods: BNC explants were cultured in DMEM and samples were divided into four groups. In group A (control); samples were only cultured in DMEM and group B; was treated with IL-1α (10 ng/ml), group C; treated with TGF-β1 (10 ng/ml) and group D; treated with IL-1α (10 ng/ml) + TGF-β1 (10 ng/ml) for 14 days. At days 3, 7 and 14 the media were collected and replaced with fresh media. Then, samples were harvested on days 3, 7 and 14 and chondrocyte morphology was assessed by invert microscopy. We used Masson's Trichrome stain to visualize collagen distribution and synthesis, and Safranin O and Alician blue to highlight the proteoglycan content.

Results: In the presence of IL-lα, most of the chondrocytes were transformed into fibroblast-like morphology with pyknotic nuclei at day 14 and proteoglycan and collagen in extracellular matrix (ECM) were destructed at this time. In the presence of TGF-β1, chondrocytes preserved their ordinary normal features. Also, TGF-β1 inhibited collagen and proteoglycan destruction and cartilage ECM showed normal characteristics.

Conclusions: IL-1α induced significant morphological changes in chondrocytes and increased the destruction of ECM. TGF-β1 could strongly preserves cartilage from IL-1α degradation effects.

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Introduction

The main feature of joint disorder is loss of articular cartilage [1, 2]. Articular cartilage is composed of chondrocyte embedded in extracellular matrix (ECM) [3, 4]. Collagens and proteoglycans presented in ECM are responsible for the load-bearing properties of articular cartilage [1]. Collagen makes up approximately 15-22% of the wet weight and contains 90-95% type II collagen fibers with a small percentage of types IX and XI [1, 2]. Destruction of cartilage involves the loss of collagens and proteoglycans. The synthesis, maintenance and degradation of ECM proteins are coordinated by chondrocytes [5]. Chondrocytes are able to synthesize new proteoglycans and to restore the cartilage completely [4]. Stimulating the chondrocytes to resynthesize ECM molecules require a complex strategy, including growth factor provision and possibly application of mechanical stimulus [6]. Transforming growth factor (TGF)-β is an important anabolic factors that stimulates event of chondrogenesis, cell proliferation, migration, adhesion, differentiation, and also cartilage development, homeostasis and repair [7, 8]. TGF-β super family has more than 35 members that contains three mammalian isoforms like TGF-β1 [9].

Previous studies had showed that TGF-β1 is a critical factor in cartilage regeneration [10]. Proteoglycan synthesis was stimulated by TGF-β1 in bovine chondrocytes depending on the differentiation stage of chondrocytes in a dose-dependent state [1]. Mabrouk et al (2008) indicated that pretreatment of knee and hip bovine joints cartilage with TGF-β (10 ng/ml) in primary monolayer cultures could induce cartilage repair in articular joints; however, in maximum doses, TGF-β1 is able to lead to inflammation [11]. It could be concluded that this growth factor maintains chondrocyte and cartilage homeostasis, but changes in receptor expression, modify the effect of TGF-β1 on chondrocyte function [12]. During the joint diseases, chondrocytes display high levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 [12]. IL-1 has the ability to stimulate chondrocytes to degrade both proteoglycan and collagen type II and produce nitric oxide, matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS) [13]. Previous studies showed that IL-lα induced significant morphological changes in cartilage, transformed most of chondrocytes into a fibroblast-like morphology, decreased chondrocytes count and induced breakdown of ECM [14-16].

Proteoglycans and collagen fibrillar network have profound effects on cartilage ECM and are obviously important to the structural integrity of cartilage [4, 17]. Chondrocytes have an important role in the pathogenesis of joint disorders, thus, elucidation of chondrocyte morphological and ECM changes could result in clearing mechanisms of tissue degradation in these diseases [4, 5, 18, 19]. The effect of TGF-β1 on regulation and control of cell growth is widely studied, but the capability of this cytokine to regulate cartilage functions such as cell death, proliferation, differentiation and ECM repair is not clearly understood [20]. Therefore, the aim of present study was to investigate the protective

effect of TGF-β1on degenerating characteristics of IL-lα in bovine nasal cartilage (BNC).

Materials and Methods

Chemicals

In this experimental study, we obtained human recombinant IL-1α from Gibco (UK-PHC0017), TGF-β1 from biolegend (US-580702). Dulbecco's modified Eagle's medium (DMEM) was also obtained from Biowest (France). Glutamine, penicillin G, streptomycin, amphotericin B, and L-ascorbic acid were all obtained from Sigma (UK).

Preparation of cartilage

Bovine nasal cartilage was obtained from adult cows after sacrificing. Nasal septum was dissected out and all samples were washed thoroughly with normal saline and cold sterile phosphate buffered saline (PBS). Perichondrium was removed from the cartilage with a sterile scalpel. The tissue was washed several times with DMEM containing 2000 U/ml penicillin G and 0.1 mg/ml streptomycin plus 2.5 µg/ml amphotericin B. The washing repeated again with the latter formula but at this time, it contained 10 fold the concentration of penicillin and streptomycin [14].

Explant culture

For explant culture, the samples were punched by a sterile 1 mm diameter punch tool. The uniform slices were cultured in serum free DMEM that contained 2000 U/ml penicillin G, 0.1 mg/ml streptomycin, 2 mM glutamine, 2.5 µg/ml amphotericin B, and 50 µg/ml L-ascorbic acid for 14 days in one 24-well sterile plate at 37˚C in a humidified atmosphere of 5% CO2 and 95% O2 [2, 5].

Experimental design

The BNC samples were divided into four groups. In group A (control), samples were cultured only in DMEM; and group B, samples were treated with IL-1α (10 ng/ml); group C was treated with TGF-β1 (10 ng/ml) and group D treated with both IL-1α (10 ng/ml) + TGF-β1 (10 ng/ml) for 14 days [14, 15]. Four wells were considered for each group. Each well contained four pieces of cartilages in 800 µl of medium and plates were incubated at 37˚C. At days 3, 7 and 14, the samples were harvested and assessed for morphology and ECM alteration by invert microscopy (LABOMED) and, light microscopy (Olympus, Japan) [14, 21]. The analysis was performed by a histologist.

Chondrocyte morphology and ECM breakdown assay

Morphological alterations of unfixed BNC explants and chondrocytes in different groups were assessed by invert microscopy at days 3,7 and 14.

Histological assessment

A number of BNC samples in different groups were collected and their histological alterations were assessed using histological method and a light microscope at days 3, 7 and 14 of culture.

Proteoglycan degradation assay

Alcian blue staining

Alcian blue was used to visualize the alterations of proteoglycans in different groups. Cartilage samples were taken out of the culture from each group at days 3, 7 and 14, fixed with formaldehyde, and processed for standard histological staining. The 6 µm sections were stained in a 3% solution of Alcian blue for 25-30

mins. [8]. Digital histographic images were captured using an Olympus light microscope (magnification 400×) [22].

Safranin O staining

Safranin O staining was also used to visualize the alterations of proteoglycans in different groups. The 6 µm sections were stained with Hematoxylin for 5 mins. and then stained with fast green solution for 5 mins. The sections rinsed quickly with 1% acetic acid solution for 10–15 seconds. After that, tissues stained in 0.1% Safranin O solution for 5 mins. [23].

Collagen degradation assay

Masson's Trichrome staining

Masson's Trichrome staining was used to visualize the alterations of collagen in different groups. At days 3, 7 and 14 a number of samples were fixed with formaldehyde, dehydrated, cleared, and embedded in paraffin wax. The 5-7 µm sections were stained with Hematoxylin, for 10 mins. and with phosphotungstic/ phosphomolybdic acid for 10 mins. and then transferred directly into fast green for 5 mins and 1% Acetic acid for 1 min. Finally, sections rinsed in distilled water, dehydrated, cleared, and coversliped [24]. It should be mentioned that the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran approved this research.

Results

Morphological assessment

Chondrocytes morphology in different groups was assessed by an invert microscope at days 3, 7 and 14. In control group that was cultured only with DMEM, chondrocytes showed round shape after 14 days of culture (Fig. 1A). In IL-lα treated group, at day 3, prominent morphological alterations in chondrocytes were begun. After 7 days, an increase in chondrocyte: matrix ratio was observed due to complete disappearance of ECM. At day 14, most of cells showed fibroblast-like morphology with a foamy-vacuolated cytoplasm and black appearance in nuclei (Fig. 1B).

In the presence of TGF-β1, the cells preserved their original spherical morphology. There were little chondrocytes with fibroblast like morphology in TGF-β1 and TGF-β1+IL-lα treated groups. The greatest amount of restoration was shown by TGF-β1 that occurred between days 7-14 (Fig. 1C and Fig. 1D).

Proteoglycan degradation in BNC cultures

Proteoglycan degradation, ECM changes and morphological alterations of explants in different groups were assessed using Alcian blue (Fig. 2) and Safranin O staining (Fig. 3).The results of Alcian blue staining showed that in group A, there was a relatively an intensive staining of proteoglycan (Fig. 2A). There were a significant correlations between the incubation day with IL-lα, proteoglycan destruction, and the disappearance of Alcian blue staining (Fig. 2B). Dark blue staining disappeared in IL-lα treated group, which indicated the loss of proteoglycan and ECM changes in this group. In TGF-β1 treated group, ECM showed an intensive staining of proteoglycan (Fig. 2C). In IL-1α+TGF-β1treated group, ECM was significantly stained with Alcian blue and increased pericellular matrix staining in this group as compared with IL-1α treated group and ECM was intact (Fig. 2D).

Results of Safranin O staining showed that in group A, there was an intensive staining of proteoglycan (Fig. 3A). In the presence of IL-lα, orange color of Safranin O disappeared which

indicated the loss of proteoglycan and ECM changes (Fig. 3B). In TGF-β1treated group, ECM showed an intensive staining of proteoglycan (Fig. 3C). In IL-1α+TGF-β1 treated group, ECM was significantly stained with Safranin O, as compared with IL-1α treated group and cartilage ECM was intact (Fig. 3D).

Collagen changes in BNC culture

Collagen degradation and ECM changes of explants were assessed in different groups using Masson's Trichrome staining at days 3,7 and 14 of culture (Fig. 4). In group A, there was a strong staining of collagen (Fig. 4A). Green staining disappeared in IL-lα treated group, which indicated collagen degradation and ECM changes (Fig. 4B). In TGF-β1treated group, ECM showed an intensive staining of collagen (Fig. 4C). In IL-1α+TGF-β1treated group, ECM was significantly stained with Masson's Trichrome staining and ECM was intact in comparison to IL-1α treated group (Fig. 4D).

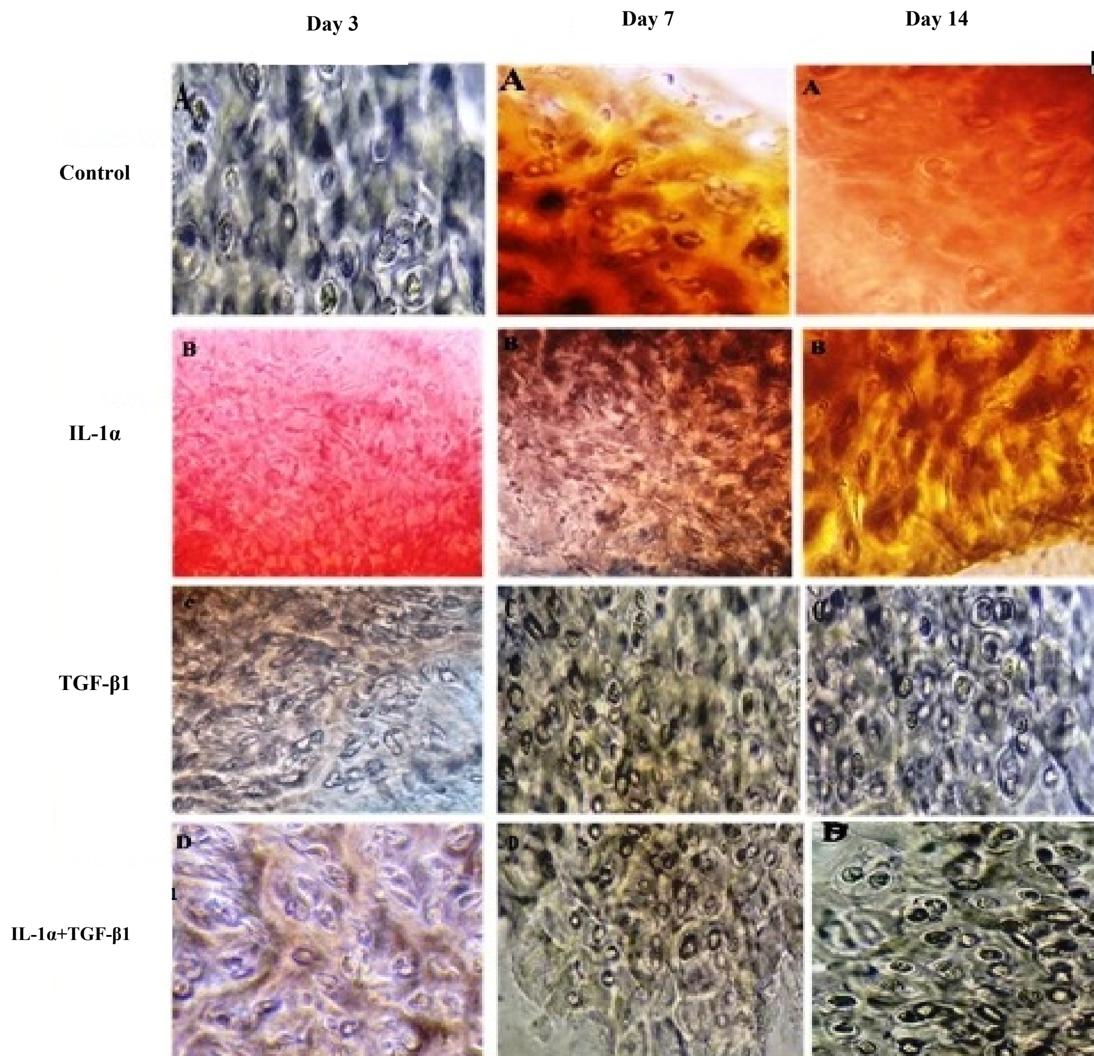

Fig. 1. Chondrocytes morphology in BNC explants at days 3, 7 and14. **(A)** Samples from control group; **(B)** explant samples cultured in the presence of IL-1α; the chondrocytes showed granular black appearance and fibroblast like morphology; and **(C** and **D)** Cartilage explants cultured in the presence of TGF-β1and IL-1α+TGF-β1, respectively. Chondrocytes preserved their normal phenotype (400×).

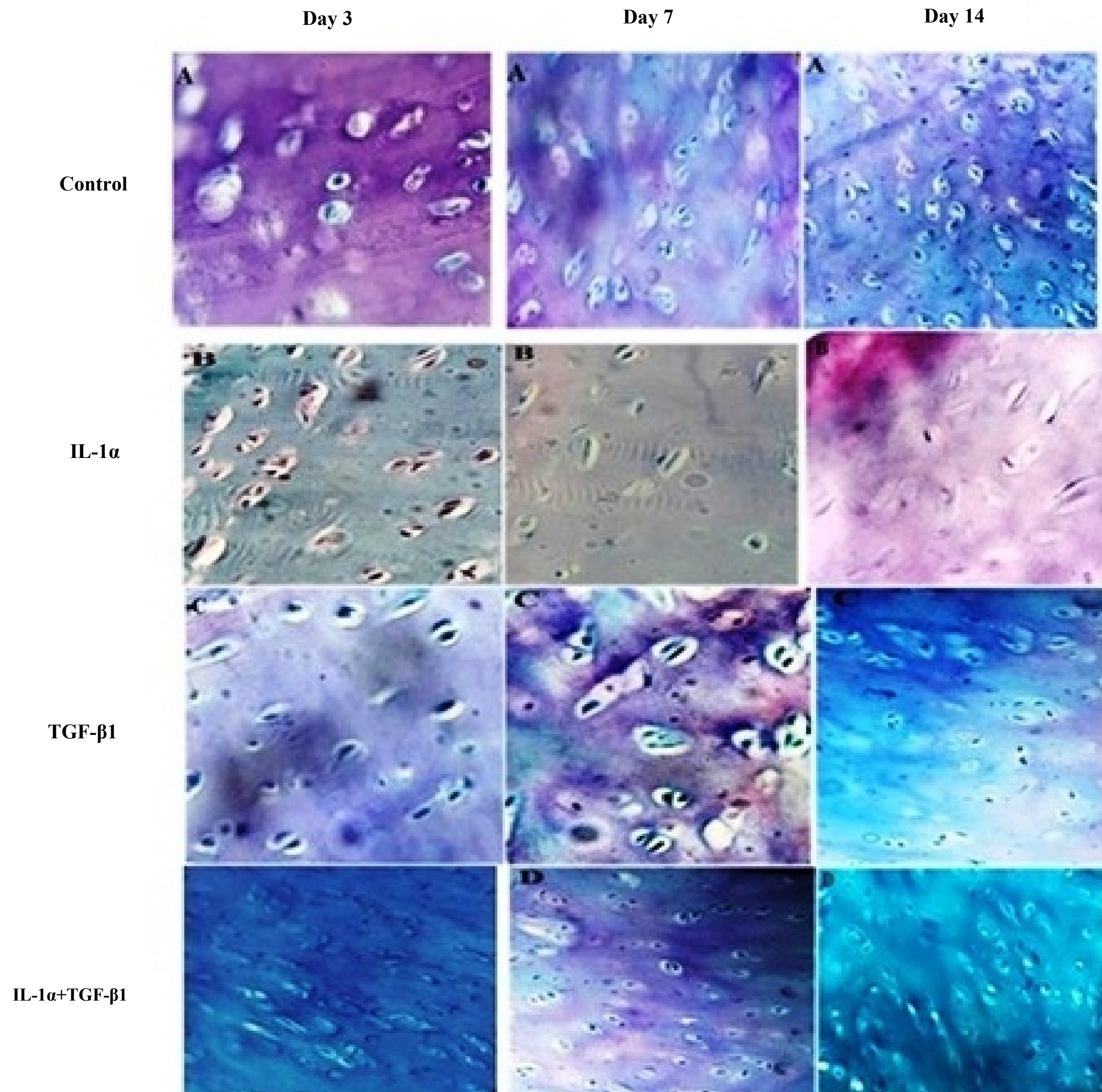

Fig. 2. Alcian blue staining of proteoglycan of BNC samples in different groups, at days 3, 7 and14. **(A)** In control group, there was relatively an intensive staining of proteoglycan; **(B)** In IL-lα treated group, dark blue staining completely disappeared and ECM was degenerated; **(C)** In the presence of TGF-β1, there was an intensive staining of proteoglycan and ECM was intact. **(D)** In the presence of IL-1α+TGF-β1, ECM stained significantly with Alcian blue as compared to group B (400×).

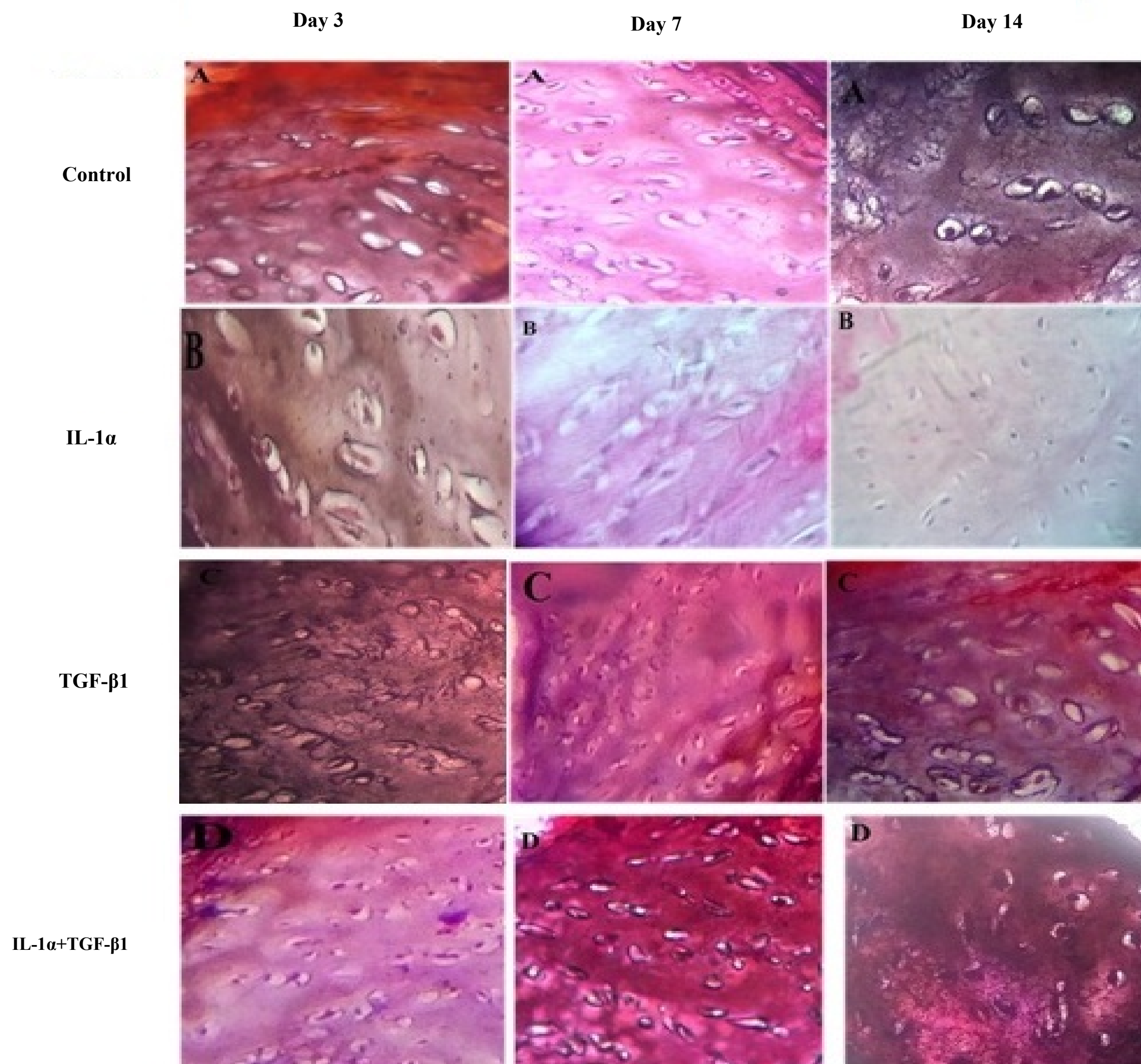

Fig. 3. Safranin O staining of BNC samples proteoglycans of in different groups, at days 3, 7 and 14 **(A)** In control group, there was relatively an intensive staining of proteoglycan; **(B)** In IL-1α treated group, (red to orange) staining completely disappeared and ECM was degenerated; **(C)** In the presence of TGF-β1, there was an intensive staining of proteoglycan and ECM was intact. **(D)** In the presence of IL-1α+TGF-β1, ECM stained significantly with Safranin O as compared to group B (400×).

Disscussion

In current study, we used TGF-β1against tissue degradation induced by IL-lα. The results showed that TGF-β1significantly preserved the normal morphological characteristics of cartilage and prevented degradation effects of IL-lα. In the presence of IL-lα, the chondrocytes showed fibroblast-like morphological changes and black point nuclei and there were decreased numbers of chondrocytes. These morphological changes were similar to Yadegari et al. [21] and Kozaki et al. reports.

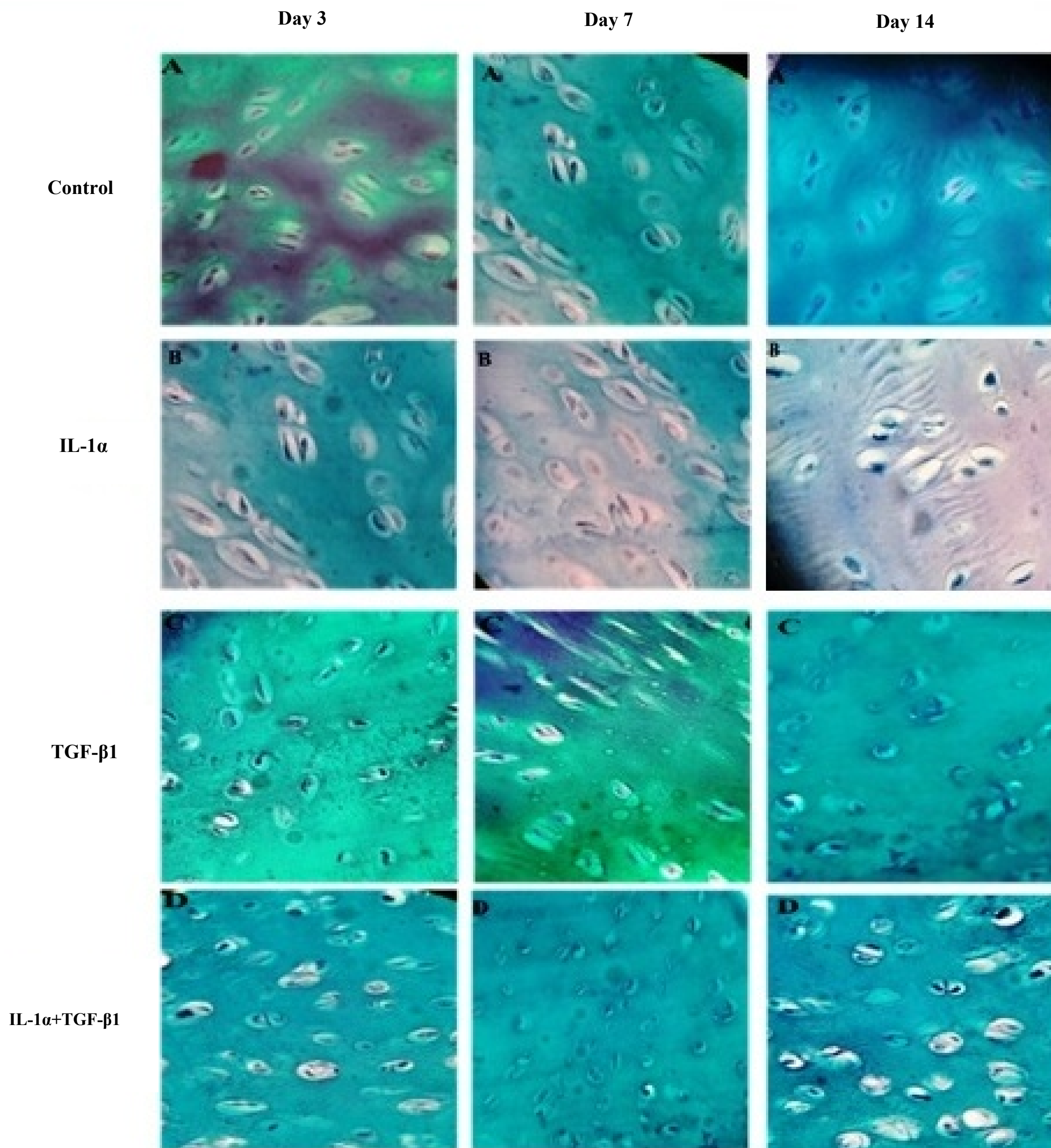

Fig. 4. Masson's Trichrome staining of BNC samples collagen of in different groups, at days 3, 7 and 14, **(A)** In control group, there was an intensive staining of collagen; **(B)** In IL-lα treated group, (green) staining completely disappeared and ECM was degenerated; **(C)** In the presence of TGF-β1, there was an intensive staining of collagen and ECM was intact. **(D)** In the presence of IL-lα+TGF-β1, ECM stained significantly with Masson's Trichrome as compared to group B (400×).

They showed that in BNC explants, IL-lα induced prominent morphological changes and most chondrocytes transformed into a fibroblast-like morphology with a granular black point appearance. Kozaci et al. [15] declared that black nuclei in BNC cultured with IL-lα (50 ng/ml) may be because of degenerating cells. The data of the current study showed that TGF-β1 preserved round morphology of chondrocytes and few numbers

of cells showed a fibroblast-like shape with pyknotic nuclei.

It has been previously demonstrated that fibroblast-like chondrocytes are dedifferentiated and the round morphology is essential to support the chondrocyte phenotype [20, 25-27]. According to these studies, TGF-β1 could prevent the dedifferentiation process and preserve the chondrocyte normal round shape as compared with IL-lα group. Our results are agreement with Yang et al. [17]. They showed that Smad3-mediated TGF-β signals are important for maintaining articular cartilage in the quiescent state by repressing chondrocyte differentiation and controlling matrix molecules synthesis. Impairment of TGF-β signals due to Smad3 disruption results in phenotypes that resembling human osteoarthritis. Their results suggested that TGF-β/Smad3 signals are essential for repressing articular chondrocyte differentiation.

Khaghani et al (2012) investigated the effect of TGF-β1 on regulation of cell growth in chondrocyte. The cells were isolated from knee articular cartilage of neonate rats and seeded at low density to obtain a fibroblast like morphology. Proliferation rate of the cells in medium with TGF-β1 supplemented was noticeably lower than control group. These results indicated that TGF-β1 stimulates chondrocyte-ECM adhesion but this effect depends on cell phenotype [28]. Although there were differences between our study and Khaghani et al reports, in regard to duration of incubation and usage of BNC explants in our study. The results of the current study also showed that TGF-β1mainatined chondrocyte round and normal morphology and prevented fibroblastic changes.

Chondrocytes in articular cartilage have a normal round shape. There are multiple interactions between the chondrocyte and ECM. These interactions are critical to the biological functions of chondrocytes, such as the synthesis and degradation of ECM components and differentiation. Chondrocytes are responsible for synthesis, maintenance and maturation of matrix, where they are embedded [10, 25, 29, 30]. It has been previously shown that in the absence of supporting matrix, chondrocytes lose their ordinary normal morphology and become fibroblast-like dedifferentiated cells [9, 23, 27].

Observations related to TGF-β1 effects on ECM alterations differ widely. There are few studies that investigated effects of TGF-β1 on chondrocyte morphology and production and preservation of ECM. The ability of TGF-β1on chondrocyte morphology and restoration of collagen type and proteoglycan is not clearly understood.

In present research, TGF-β1 significantly decreased BNC proteoglycan degradation induced by IL-1α. Increased pericellular matrix staining showed a new proteoglycan synthesis around chondrocytes and possibly reflects the repairing effects of TGF-β1, in TGF-β1 and TGF-β1+ IL-1α treated groups. There was a parallel correlation between proteoglycan degradation and changes in chondrocyte morphology.

In current study, protective effects of TGF-β1 on proteoglycan content in BNC explant culture were similar to results of Molares et al.

[31]. They studied TGF-β effects on bovine articular cartilage organ culture and showed that TGF-β stimulated synthesis of proteoglycans in a dose-dependent manner, reached saturation at 10 ng/ml. TGF-β (10 ng/ml) caused a significant increase in proteoglycan synthesis and decrease in catabolism of proteoglycan in comparison to controls [31].

In present study, TGF-β1 significantly decreased BNC collagen degradation induced by IL-1α. Increased matrix staining showed a new collagen synthesis and possibly reflects the repairing effects of TGF-β1. Our results are agreement with Hui et al. [32]. They used IL-1 (1 ng/ml), IL-1 (0.2 ng/ml)+OSM (2 ng/ml) to induce rapid release of collagen from BNC in culture. TGF-β (1–25 ng/ml) caused dose-dependent inhibition of IL-1 or IL-1+OSM stimulated collagen release and were able to block this release of collagen from the tissue, and to reduce the secretion of collagenases while maintaining the level of tissue inhibitor of metalloproteinase-1 (TIMP-1) in BNC at day 14. Expression of MMPs mRNA was assessed by Northern blot analysis. In present study, we also investigated the effects of IL-1α and TGF-β on ECM and collagen distribution and synthesis of BNC by light microscope histological assessment, and Masson's Trichrome staining at designated time points. Although we used higher doses of IL-1α for degradation of ECM, our results were consistent with Hui et al. In present study, results showed that TGF-β1 inhibited collagen destruction and cartilage ECM was intact. There was a strong staining of collagen. In another study, Hui et al. showed that TNFα was able to promote collagen breakdown in bovine cartilage in explant culture. This release was depended to MMPs and TGF-β1 or insulin-like growth factor 1 could prevent or reduce the secretion of collagenase enzymes, and induce TIMP production. Their study showed for the first time that TGF-β1 could protect cartilage against TNF induced destruction [33]. Our results are agreement with Hui et al reports.

Conclusions

In current work, at day 3, morphological alterations and damages induced by IL-lα were begun. The highest amount of damages induced by IL-lα occurred at day 7, but the greatest amount of restoration is by TGF-β1 occured between days 7-14. The present research results showed that TGF-β1 restored and stimulated production of collagen and proteoglycan and preserved chondrocyte original morphology. Chondrocytes have an important role in the pathogenesis of joint disorders; thus, elucidation of chondrocyte morphological and ECM changes could result in clearing mechanisms of tissue degradation in these diseases. In present study, TGF-β1 preserved chondrocyte ordinary normal features and inhibited collagen and proteoglycan destruction and also ECM showed normal characteristics. It seems that TGF-β1 is a perfect choice for treatment in regard to joint disorders researches.

Conflict of Interests

The authors have no conflicting financial interests.

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