

Combination Effects of Prednisolone and Interleukin-4 Protect Bovine Nasal Cartilage Explants from Interleukin-1 α Induced Degradation

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ABSTRACT

Background: Current treatments for joint diseases are moderately successful, but unfortunately are associated with significant side effects. This study was undertaken to investigate the combination effects of IL-4 and prednisolone on tissue characteristics and production of matrix metalloproteinase-1 (MMP-1) in IL-1 α -treated bovine nasal cartilage (BNC) explants. **Methods:** BNC explants were cultured in DMEM with IL-1 α (10 ng/ml), IL-4 (50 ng/ml) and prednisolone (1 or 1,000 nM) at the same time for 28 days. At days 3, 7, 14, 21 and 28, the media were collected and replaced with fresh media, and the removed media were stored at -20°C. The alterations of tissue characteristics were assessed by using histology techniques. Western-blot method was used to determine the effects of IL-4 and prednisolone combination on MMP-1 production. The cell viability was evaluated by using lactate dehydrogenase assay test. **Results:** In the presence of IL-1 α alone, most chondrocytes were transformed into fibroblast-like morphology with pyknotic nuclei at day 28. In addition, a clear band of MMP-1 and extracellular matrix (ECM) degradation were observed. In combination of IL-4 and prednisolone, chondrocytes preserved their ordinary normal features. MMP-1 band formation was completely inhibited and ECM absolutely showed normal characteristics. IL-4 and prednisolone did not show cytotoxicity effects on BNC explant culture. **Conclusion:** This combination can strongly preserve cartilage from degradation features and the data possibly suggest that the combination of IL-4 and prednisolone could be a candidate for alternative therapy in joint diseases. *Iran. Biomed. J.* 15 (4): 143-150, 2011

Keywords: Chondrocytes, Prednisolone, Interleukin-4 (IL-4), Matrix metalloproteinase-1 (MMP-1)

INTRODUCTION

Joint diseases, such as rheumatoid arthritis and osteoarthritis affect a significant proportion of the population around the world. The main features of these diseases are loss of articular cartilage and inflammation that lead to severe pain and disability [1, 2]. Articular cartilage is a simple connective tissue composed of the only one cell type called chondrocyte, embedded in an abundant extracellular matrix (ECM) [3, 4].

Increased levels of matrix metalloproteinases (MMP) result in degradation of cartilage ECM components, destruction of bone, and joint abnormalities. Three mammalian collagenases include interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase 3 (MMP-13) can specifically cleave triple helical collagen [5-9]. Previous studies

have reported that MMP-1 is one of the predominant enzymes that mediate fibrillar collagen degradation in bovine nasal cartilage (BNC) explant culture system [4, 6]. It has been also demonstrated that identification of MMP regulation mechanisms in cartilage may lead to the development of new treatment for arthritis [9-11]. MMP-1 is upregulated by pro-inflammatory cytokines such as IL-1 and tumor necrosis factor α [4-6].

A number of studies have shown the critical role of anti-inflammatory cytokines such as IL-4 in arthritis diseases. IL-4 is a multi-functional T-cell derived cytokine that down-regulates the production of pro-inflammatory cytokines [12, 13]. Although previous studies have reported the effects of IL-4 on cartilage and synovial tissues, the exact mechanism of IL-4 effects on MMP production has not been well-established [14-16].

Glucocorticoids, as commonly accepted anti-

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inflammatory agents, can decrease macrophage phagocytosis and IL-1 secretion, resulting in inhibition of collagenase and releasing of lysosomal enzymes [17, 18]. Because these drugs are efficient anti-inflammatory compounds, they usually improve the clinical signs of swelling and providing pain relief. In a recent study, Lu *et al.* [19] showed that glucocorticoids treatment effectively abolished the catabolic effects which exerted by the pro-inflammatory cytokines in bovine and human articular cartilage explants. A major concern following usage of glucocorticoids is that the beneficial effects are outweighed by deleterious effects on articular cartilage, and the bone (osteoporosis). Prednisolone is one of these well-known anti-inflammatory drugs [12, 17, 20].

In a previous study, the systemic effects of IL-4 and prednisolone treatment were assessed in established murine collagen-induced arthritis. Synergistic suppression of both arthritis severity and cartilage degradation was reported when combination of IL-4 and prednisolone was applied. They also observed neither IL-4 nor prednisolone by itself were effective, and proposed that a further study is needed to resolve the other mechanisms of these combination effects [12].

Although there are several studies that have focused on the effects of cytokines and glucocorticoids, their exact activity mechanisms on cartilage degradation have been less characterized [18, 20]. To our knowledge, there is no proper studies evaluated the combination effects of IL-4 and prednisolone on BNC explants. Therefore, we have undertaken this study to investigate the protective effects of both IL-4 and prednisolone on degenerating characteristics of IL-1 α -induced cartilage.

MATERIALS AND METHODS

Cartilage preparation. The procedure was applied as we described previously [21]. BNC was obtained from adult animals at a local abattoir after sacrifice. The tissue was washed thoroughly with PBS containing 10 \times PEST (2,000 U/ml penicillin and 0.1 mg/ml streptomycin) and DMEM containing 1 \times PEST (2.5 μ g/ml amphotericin B), respectively. Then, the tissue was dissected with a scalpel and perichondrium-free samples were prepared.

Explant preparation and cartilage degradation assay. The BNC was punched using a sterile 2-mm diameter punch tool. The uniform slices were cultured in serum-free DMEM supplemented with 2,000 U/ml penicillin G, 0.1 mg/ml streptomycin, 2 mM glutamine, 2.5 μ g/ml amphotericin B and 50 μ g/ml ascorbic acid 2-phosphate (Vitamin C) in a humidified atmosphere of

5% CO₂, 95% O₂ in a 24-well sterile plate at 37°C for 28 days [6, 21]. Then, explants were treated with IL-1 α (10 ng/ml, PHC0017, Gibco, USA) [21], IL-4 (50 ng/ml, PHC0045, Gibco, USA) [4] and prednisolone (1 or 1000 nM, Sigma, P6004) [18], respectively as the test groups and explant cultured in DMEM alone as a control group. The groups classified into: A, control group; B, IL-1 α ; C, IL-4 + IL-1 α ; D, IL-1 α + prednisolone (1 nM); E, IL-1 α + prednisolone (1,000 nM); F, IL-1 α + IL-4 + prednisolone (1 nM) and G, IL-1 α + IL-4 + prednisolone (1,000 nM) for 28 days. All media were replaced with fresh media containing identical test reagents to day 1 and the media from days 3, 7, 14, 21 and 28 were stored at -20°C until process [6].

Histology assessment. To survey degradation of ECM and chondrocyte morphology, cartilage explants from each treatment were taken out of culture on the appropriate day. The samples were fixed in formaldehyde and tissue characteristics were determined by using histology method with hematoxylin and eosin [22] in accordance with routine laboratory procedures. All of the sections were studied by using an Olympus light microscopy (Olympus, Japan, magnification 40 \times).

Western-blotting. Blotting for MMP-1 was performed on culture medium from days 3 to 28 of culture. The medium samples were analyzed by SDS-PAGE on 12% polyacrylamide gels electrophoresis under reducing conditions. Medium from both control and test groups were thawed in room temperature. Then, 20 μ l of medium samples were dissolved in sample buffer and boiled with 2-ME (Merck) for 5 min according to the standard protocols using the Laemmli buffer system [23] and our previous work [21]. After doing standard protocol, the membrane was incubated with primary antibody, a rabbit anti-human MMP-1 (1:250, sc-6837, Santa Cruz Biotechnology Inc, USA) in room temperature for 8 h. Then, washing was carried out three times with Tris-buffered saline + Tween-20 (TBST) for 15 min and the membrane was incubated with the secondary polyclonal antibody, goat anti-rabbit IgG-HRP (1:2000, sc-2004, Santa Cruz Biotechnology Inc, USA) in TBST for 1 h. The bands of MMP-1 were detected by using diaminobenzidine tetrahydrochloride (Dako) as substrate [6, 24]. Marker MW (45-250 kDa, Sigma, USA) was used to determine the MW of MMP-1.

Cytotoxicity assay. Cytotoxicity assay was designed as a colorimetric alternative to quantitate cytotoxicity/cytolysis based on the measurement of lactate dehydrogenase (LDH) released from non-viable cells into the medium. The Cytotoxicity Detection Kit

(Roche Diagnostic) was used. All tests were performed in triplicate in test, low control (LDH activity released from the untreated-normal cells) and high control (the maximum releasable LDH activity in the cells) groups. In high control group, the maximum amount of releasable LDH enzyme activity was determined by lysing the cells with Triton X-100 (final concentration: 1% Triton X-100). At this concentration, Triton X-100 did not affect the LDH activity. Then, samples from A, control group; B, IL-1 α ; C, IL-4 + IL-1 α ; E, IL-1 α + prednisolone (1,000 nM) and G, IL-1 α + IL-4 + prednisolone (1,000 nM) were used (the maximum dose of IL-1 α , IL-4 and prednisolone were used). The medium of each group (100 μ l/well) was carefully removed and transferred into corresponding wells of a 96-well flat-bottom micro plate. To determine the LDH releasing into the medium, a 100- μ l reaction mixture was added to each well and incubated at 15 to 25°C for up to 30 min. The absorbance of the samples was measured at 490 or 492 nm using an ELISA reader. The average absorbance values of the triplicates were calculated and the resulting values were substituted in the following formula (Kit Roche Diagnostic):

$$\text{Cytotoxicity (\%)} = \frac{\text{Exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Statistical analysis. Statistical significance of difference was assessed with one-way ANOVA by SPSS for Windows (version 15) followed by post hoc Tukey test. A value of $P < 0.05$ was considered significant.

RESULTS

In this study, the combination effects of IL-4 and prednisolone on tissue characteristics and MMP-1 production in IL-1 α -treated BNC explants were investigated.

Cartilage tissue evaluation. The morphology of chondrocytes was evaluated by using hematoxylin and eosin staining after 28 days of culture (Fig. 1). In control group cultured in DMEM alone, chondrocytes showed a round and polygonal shape (Fig. 1A). Histological sections of cartilage explants in the presence of IL-1 α showed the flattened fibroblast-like morphology with pyknotic nuclei, and ECM degradation was prominent (Fig. 1B). In the IL-1 α + IL-4-treated group, chondrocytes preserved their basic morphology in an isogenic group (Fig. 1C). In the presence of IL-1 α + prednisolone (1 nM), chondrocyte morphology was identical to IL-1 α alone group (Fig. 1D). In IL-1 α + prednisolone (1,000 nM)-treated group, chondrocyte phenotype changes in BNC

explants were partially decreased. In this group, some cells showed fibroblast-like morphology changes and a number of cells showed round shape morphology (Fig. 1E). In F and G groups, which the difference between them was prednisolone concentration, the most chondrocytes preserved their typical characteristics. ECM was intact and a few numbers of fibroblast-like chondrocytes with pyknotic nuclei were observed (Figs 1F and 1G).

Determination of MMP-1 production in media. The medium samples of different groups from days 3, 7, 14, 21 and 28 were collected and subjected to reducing Western-blot analysis. According to optimum results, samples at day 21 were analyzed by Western-blotting. No band of MMP-1 was detected in control group. IL-1 α alone stimulated a very clear band of MMP-1 (84 kDa) from BNC explants. In the presence of IL-1 α + IL-4, a weak band of MMP-1 was detected. In the IL-1 α + prednisolone (1 nM)-treated group, MMP-1 band was identical to IL-1 α alone group and a clear band was detected. In the IL-1 α + prednisolone (1,000 nM)-treated group, MMP-1 band was partially reduced. In combination of IL-1 α + IL-4 + prednisolone (1 nM), a very weak MMP-1 band was detected. The combination of IL-4 with 1,000 nM of prednisolone showed a synergistic reduction of MMP-1 and no band of MMP-1 was detected (Fig. 2).

Cytotoxicity assay. The LDH releasing in the culture medium as a marker for disruption of the cell membrane integrity and an index of cell death was measured at day 28. The other groups were compared with control and IL-1 α -treated group. As shown in Figure 3, difference between control and IL-1 α was significant ($P < 0.05$), and between control and other groups was not significant ($P > 0.05$).

DISCUSSION

In the present study, for the first time, we have used IL-4 and prednisolone at the same time in BNC explant culture to protect cartilage from degradation effects of IL-1 α . The data of the current study show that the combination of IL-4 and prednisolone could preserve cartilage from apoptotic and degenerating manifestations.

To obtain a pattern of degradation induced by IL-1 α , a time point from the previous studies was applied [6, 22, 24] and based on our study was adjusted. BNC explants were treated with IL-1 α and applied as a model of cartilage degradation as we applied previously [21]. The chondrocytes showed all of the apoptotic features such as pyknotic nuclei and fibroblast-like morphology changes. The MMP-1 production into the culture

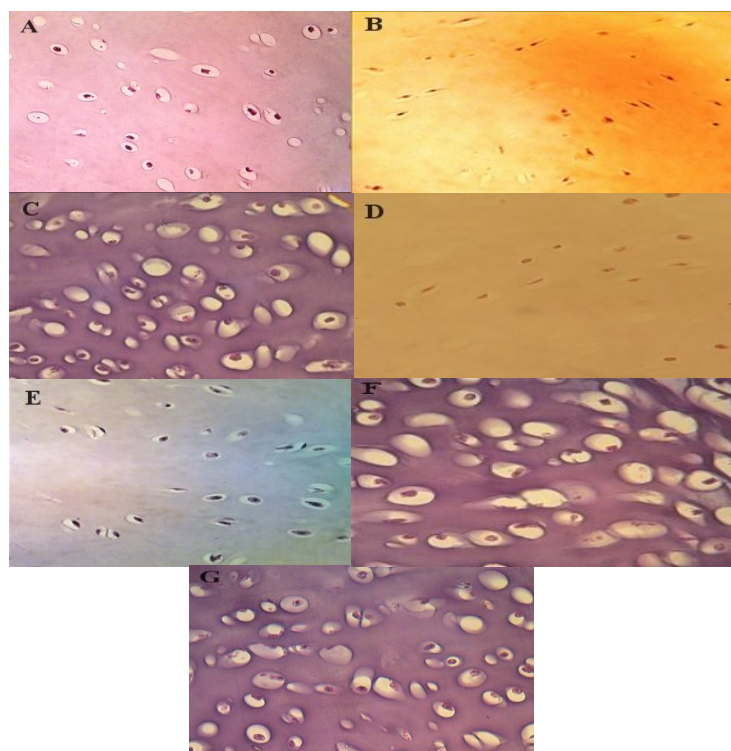


Fig.1. Morphological alterations of BNC explants in different groups at the end of experiment hematoxylin and eosin staining. **(A)** In control group, most chondrocytes preserved their ordinary shape; **(B)** in the presence of IL-1 α , flattened fibroblast-like morphology chondrocytes with pyknotic nuclei were observed and ECM degradation was also prominent; **(C)** in the presence of IL-1 α + IL-4, chondrocytes preserved their basic phenotype in an isogenic group; **(D)** in the presence of IL-1 α + prednisolone (1 nM), prominent chondrocyte morphology changes were observed; **(E)** in IL-1 α + prednisolone (1,000 nM)-treated group, a number of chondrocytes were in normal phenotype and a number of cells showed fibroblast-like morphology and ECM partially showed normal characteristic; **(F)** in combination of IL-1 α + IL-4 + prednisolone (1 nM), the most chondrocytes preserved their typical normal phenotype and ECM was intact. Also, a few numbers of fibroblast-like chondrocytes with pyknotic nuclei were observed, and **(G)** chondrocyte morphology in IL-1 α + IL-4 + prednisolone (1,000 nM)-treated group was the same as F group (40 \times).

medium was in the highest level at day 21. According to the previous studies [6, 22], this character was considered as a criterion for cartilage degradation. The MMP-1 band was the same as what detected by Kozaci *et al.* [6]. They have treated BNC explants with IL-1 α (50 ng/ml) for 2-4 weeks and suggested a correlation between MMP-1 releasing into the medium and chondrocyte morphological changes. In consistent with Kozaci *et al.* [6], the results of morphology and molecular assessments in the present study suggested a parallel and relevant association between MMP-1 releasing into the medium and ECM degradation and chondrocyte morphology alteration at day 21 of culture.

Some studies have examined the interactions between catabolic and anabolic factors on MMP expression and morphology changes in chondrocytes [25-28]. Because there are few studies that have investigated protective effects of IL-4 on chondrocyte morphology, the present study concentrated on these effects. In the current study, when BNC explants were

treated with IL-1 α and IL-4, all of the cells showed an ordinary phenotype and a round shape in an intact matrix. It has been previously reported that chondrocytes with fibroblast-like morphology are dedifferentiated, and the round morphology is essential to support the chondrocyte phenotype [29, 30]. Treatment of BNC explants with IL-4 modulated the dedifferentiation process and preserved the chondrocyte normal morphology.

In the present study, chondroprotective effects of IL-4 in BNC explant culture were similar to the data of Shimizu *et al.* [31]. They have shown that primary cell culture of rat articular cartilage in the presence of IL-4, chondrocytes preserved their original characteristics. They also reported that it is unclear how IL-4 protects matrix degradation against excessive mechanical stress. We have demonstrated that IL-4 affects both chondrocyte morphology and cartilage ECM. It seems that these alteration effects are related to down-regulation of MMP-1 production.

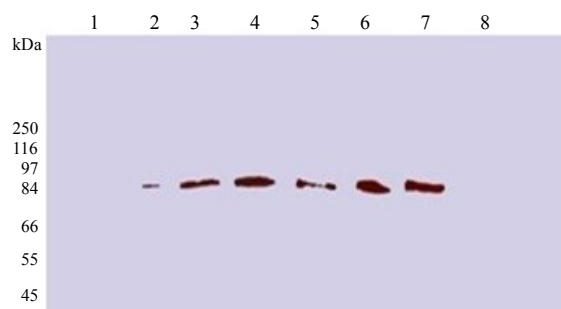


Fig. 2. Western-blot with an anti-peptide for MMP-1 in different groups at day 21. Position of MMP-1 was determined by using polyclonal antibodies. Lane 1, media taken from IL-1 α + IL-4 + prednisolone (1,000 nM)-treated group, and no band of MMP-1 was detected; Lane 2, media taken from the combination of IL-1 α + IL-4 + prednisolone (1 nM) and a very weak MMP-1 band was detected; Lane 3, media taken from IL-1 α + prednisolone (1,000 nM), and MMP-1 band was partially reduced; Lane 4, media taken from IL-1 α + prednisolone (1 nM), and a significant MMP-1 band was seen; Lane 5, media taken from IL-4 + IL-1 α and a weak band of MMP-1 was detected; Lanes 6 and 7, media taken from two different samples of IL-1 α alone, and a significant MMP-1 band was detected (84 kDa) and Lane 8, control group and no band of MMP-1 was detected.

Increased MMP production results in degradation of cartilage ECM components [5-9]. In the presence of IL-1 α + IL-4, MMP-1 band was clearly decreased that was in agreement with Cleaver *et al.* study [4]. In their study, BNC explants were treated with IL-1 α (1 ng/ml) and oncostatin M (10 ng/ml), and collagenolytic activities were determined by bioassay and Northern-blot analysis. Although we used IL-1 α alone for stimulating cartilage, and Western-blot analysis for MMP-1 detection, the results confirmed that previous data.

Previous studies have reported that glucocorticoids such as prednisolone could partially preserve the ordinary chondrocyte morphology [32]. Therefore, for studying the proper effects of prednisolone on chondrocyte morphology, two different doses of prednisolone were applied in the current work. Although in the higher dose (1,000 nM) of prednisolone, a number of chondrocytes preserved their normal features, in the lower dose (1 nM), most chondrocytes showed fibroblast-like morphology and ECM degradation was partially observed. These results revealed that low-dose prednisolone did not show visible protective effects on chondrocyte morphology changes, which induced by IL-1 α , but high-dose prednisolone showed partially protective effects on cartilage morphology. These data prompt us to combine prednisolone with the other protective agent(s) to reinforce chondrocyte morphology preservation.

Previous studies have reported that glucocorticoids have different effects on MMP releasing under *in vitro* conditions. It has been also reported that a number of glucocorticoids such as prednisolone, dexamethasone and triamcinolone affect the cartilage components and exert a panel of very different features [18, 33]. Richardson and Dodge [32] showed a variety of glucocorticoids, such as dexamethasone, prednisolone and triamcinolone can strongly down-regulate different MMP and ferritin mRNA levels in catabolic cytokines-treated equine articular chondrocytes. Decrease of MMP in their study was in consistent with the results of the present study. In addition, in their study, these glucocorticoids increased link protein and fibronectin mRNA, but did not affect the biglycan and decorin. They also reported that although chondrocytes were phenotypically maintained, high doses of glucocorticoids significantly decreased the expression of type II collagen by isolated articular chondrocytes [32]. Their findings are consistent with the previous claim that glucocorticoids may be deleterious to articular cartilage [18]. Regarding the above data in the present study, the combination of IL-4 and prednisolone was used to test and evaluate a low-dose prednisolone and thereby, decrease unwanted side effects of prednisolone.

In another study by Augustine and Oleksyszyn [18], it has been reported that a variety of glucocorticoids, such as clobetasol propionate, fluocinolone acetonide

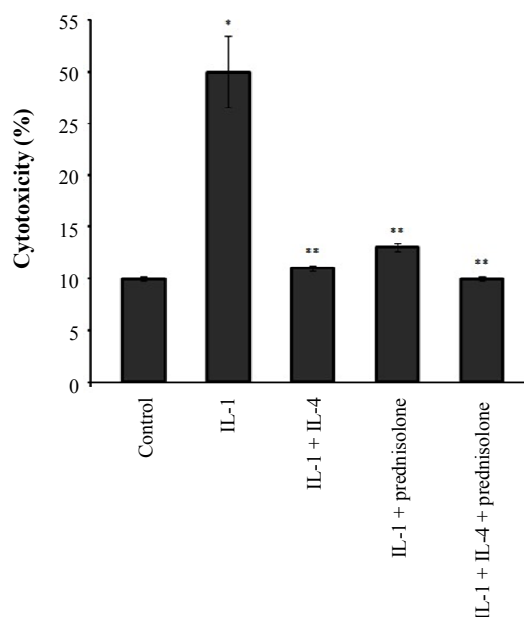


Fig. 3. Comparison of cytotoxicity assay in BNC explants after exposure to IL-1 α , IL-4 and prednisolone for 28 days. The concentrations of IL-1 α , IL-4 and prednisolone were 10 ng/ml, 50 ng/ml and 1,000 nM, respectively. * P <0.05 compared with control, and ** P <0.05 compared with IL-1 α .

21-acetate, prednisolone, triamcinolone and triamcinolone hexacetonide did not show protective effects on cartilage degradation in the presence of IL-1 α alone on bovine articular cartilage. But, when degradation was induced by IL-1 α + human plasminogen, all above glucocorticoids showed significant inhibition on MMP. They have suggested that inhibition of cartilage degradation by glucocorticoids may be due to down-regulation of urokinase plasminogen activator activity that is the first enzyme in the activation process of pro-MMP by the fibrinolytic system [18]. In this work, in the presence of IL-1 α + prednisolone, MMP-1 production was partially reduced. The existence of very weak band of MMP-1 in the presence of IL-4 with low-dose prednisolone, and no band of MMP-1, in the presence of IL-4 and high-dose prednisolone, possibly suggested that prednisolone also reduced IL-1 α effects on MMP-1 protein levels. The results of the present study are not in consistent with Augustine and Oleksyszyn's results [18] because they used different methods for detection of MMP. In addition, the other possible explanation for this discrepancy might be because of duration of incubation, or using BNC explants in our study.

This study also showed that prednisolone could reduce MMP-1 production in the presence of IL-1 α , but this reduction effect was lower compared with IL-4. Therefore, it is concluded that the combination of IL-4 and prednisolone relatively decrease MMP-1 production in BNC explants. Joosten *et al.* [12] have used a collagen-induced arthritis in murine as a disease model. They applied a systemic model treatment by using IL-4 and prednisolone, and suggested that combined IL-4 with steroid treatment may provide a safe, anti-inflammatory and anti-destructive therapy in human rheumatoid arthritis.

In the present study, the combination of IL-4 and prednisolone provide safe and protective effects on cartilage degradation induced by IL-1 α . Therefore, Joosten's results [12] are in agreement with those of the present study. In addition, to determine whether the cell shape changes affect cell viability or not, LDH levels into the medium were measured in the presence of cytokines and prednisolone by Cytotoxicity Detection Kit (11644793001, Roche Diagnostic, Germany) [4, 22]. The present toxicity assay data indicated that neither IL-4 nor prednisolone showed distinguishable toxic effects on chondrocytes in explants at the sampling points of our study. However, in the presence of IL-1 α , LDH level was increased compared with control.

Although in combination of IL-4 and high-dose prednisolone, MMP-1 band completely disappeared, in the presence of IL-4 and low-dose prednisolone, MMP-1 band was reduced more significantly in comparison to IL-4 + IL-1 α group. Morphological

assessment explained that IL-4 has significant protective effects on chondrocyte morphology in comparison with prednisolone. The results also demonstrated that treatment of explants with IL-4 and prednisolone with both low and high doses at the same time significantly preserved the cartilage normal morphological characteristics. A concluding remark from this study is that IL-4 in combination with low-dose prednisolone provides an intriguing therapeutic option. This might offer an alternative to the use of prednisolone alone, because it can circumvent the unwanted side effects of the drug such as steroid-induced osteoporosis.

A better understanding of the mechanisms by which this combination differentially regulate MMP expression in human chondrocytes could provide a valuable insight into new therapeutic strategies aimed to the prevention of cartilage destruction. In general, further studies are required to clear the proper characteristics of the combination effects of IL-4 + prednisolone or other glucocorticoids with similar characteristics. It may confirm the hypothesis that treatment with combination of IL-4 and prednisolone could offer an alternative therapy in joint diseases.

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