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## Effect of Bone Morphogenetic Protein-2 on Normal and Osteoarthritic Human Articular Chondrocytes

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**Abstract:** In this study, we investigated whether Bone Morphogenetic Protein-2 (BMP-2) could modulate dedifferentiation, apoptosis and proliferation capacity in the normal and OA cultured chondrocytes. The articular chondrocytes from normal (n = 4) and OA (n = 4) cartilages were harvested separately. The chondrocytes were cultured in monolayer in the presence of 100 ng mL<sup>-1</sup> BMP-2 and 1% FBS as a test group and 1% FBS alone as a control group. Then, the chondrocytes were harvested and assessed for morphology with invert microscopy, proliferation by using MTT-assay and apoptosis with caspase-3 immunocytochemistry. The results indicated that the normal and the most OA chondrocytes showed round and polygonal appearance with chondrocyte-like morphology in BMP-2 treated groups after 6 days. The MTT proliferation test didn't show significant difference between test and control groups. The OA cells showed proliferation rate higher than the normal cells and significant difference in the presence of BMP-2 was observed (p<0.05). Positive immunostaining of caspase-3 in test and control groups was 1 and 20% in normal and 30 and 43% in OA groups, respectively. The percentage of apoptosis was reduced in the presence of BMP-2. In conclusion, it appears that BMP-2 involves in suppression of dedifferentiation and apoptosis processes of cultured human chondrocytes.

**Key words:** Chondrocyte, BMP-2, proliferation, apoptosis, osteoarthritis

### INTRODUCTION

Osteoarthritis (OA) is the most common form of joint disease. It affects approximately 15% of the world's adult population with pain and disability in joint (Cole and Kuettner, 2002). It is characterized by progressive degeneration of articular cartilage (Hedbom and Hauselmann, 2002). Some treatments are directed to decreasing the symptoms and slowing the progress of osteoarthritis. These treatments do not affect repair of cartilage because the cartilage has not a vascular system and it has limited repairing capacity. To date, the use of growth factors, cell replacement and surgical intervention have been devised to repair damaged articular cartilage.

Transforming Growth Factor (TGF)- $\beta$  family and related Bone Morphogenetic Protein (BMP) family are the most potent of growth factors that involve in chondrocyte biosynthesis and cartilage repairing (Hedbom and Hauselmann, 2002). The TGF $\beta$  superfamily consists of more than 40 polypeptide growth factors and it is

expressed in human OA cartilage (Uchino *et al.*, 2000). Bone morphogenetic proteins (BMPs), members of this superfamily, involves in bone healing as well as in cartilage metabolism in adults and among its members, BMP-2 induces synthesis of extracellular matrix by chondrocytes and therefore cartilage growth (Jorgensen *et al.*, 2001). Fukui *et al.* (2003) showed that BMP-2 is produced by OA and normal chondrocyte. It could stimulate synthesis of collagen II and proteoglycan but probably not in sufficient amount to inhibit the activities of proinflammatory cytokines in OA.

In addition, cell therapy and tissue engineering are another strategy for cartilage repairing. Autologous Chondrocyte Implantation (ACI) is a method of cell therapy. Lindahl *et al.* (2001) indicated that ACI has provided an excellent clinical outcome in the repair of chondral defects. In ACI method, the autologous chondrocytes are isolated from a small biopsy in a low weight-bearing area and expanded in monolayer culture to achieve a proper amount of cells to fill a defect. Then

these cells either directly injected into the defect or used to engineer implantable grafts (Sittinger *et al.*, 1999). Therefore, the key factors in ensuring the success of ACI are the morphology, proliferation and apoptosis of chondrocytes.

A number of studies have found when human chondrocytes are grown in a monolayer culture, they proliferate but the round chondrocytes morphology transform into flattened fibroblast-like cells (dedifferentiation) and they change in biochemical and genetic characteristics. They showed that the dedifferentiation of chondrocytes do not express the specialized proteins of cartilage including type II collagen and aggrecan (Kolettas *et al.*, 1995). The dedifferentiation process is a major restriction for cell therapy or tissue engineering of destructive cartilage. Krawczak *et al.* (2008) showed that the BMP-2 could prevent dedifferentiation of rabbits' chondrocytes in monolayer cultures for up to 3 weeks. However, the application of BMP-2 for chondrocyte differentiation is under investigation (Oshin and Stewart, 2007) and the effect of BMP-2 on the chondrogenic phenotype of human articular chondrocytes has been less characterized. Therefore, we applied BMP-2 on the human normal and OA articular chondrocytes and compared metabolic activities such as morphology, proliferative abilities and apoptosis of them. These data probably could be used for ACI method and treatment of OA.

## MATERIALS AND METHODS

**Chemicals:** Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, Fetal Bovine Serum (FBS), penicillin/streptomycin, amphotericin B, L-glutamine, Collagenase type II, Trypsin, hrBMP-2 (human recombinant bone morphogenetic protein-2), Collagen type I, DMSO (dimethyl sulfoxide) and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide] were purchased from Sigma, USA. Anti-caspase-3 and mouse ABC staining system were purchased from Santa Cruze biotechnology, Inc Europe.

**Isolation of chondrocytes:** Samples of normal and OA human cartilage from the femoral head were obtained during surgery from patients with osteoporotic femoral neck fractures. Normal cartilages were four individuals (one male and three females, range 67-87, mean 77 years) and OA cartilages were four patients (two male and two female, range 70-86, mean 78 years.). None of the subjects had a clinical history of inflammatory or non-inflammatory joint disease, or chronic systemic inflammatory disease. This study was performed in Anatomy department's Cell

Culture Lab. of Ahvaz Jondishapour University during autumn 2008 and was approved by the local ethics committee of Ahvaz Jondishapour University of Medical Sciences.

Femoral heads were immersed in antibacterial solution (phosphate-buffered saline (PBS) solution containing 100 U mL<sup>-1</sup> of penicillin, 100 mg mL<sup>-1</sup> of streptomycin, 0.25 mg mL<sup>-1</sup> of amphotericin B and 2 mM L-glutamine) and transported to the cell culture laboratory. The OA cartilages were examined and changes of osteoarthritis, if present, were graded 0-4 according to the classification of Collins and McElligott (1960). Sections of normal and OA cartilage were removed carefully and pooled in antibacterial solution and slices of them were fixed in formaldehyde for histological assessment. The remaining of cartilage was minced into small pieces (1-3 mm<sup>3</sup>) and washed in antibacterial solution. After rinsing, cartilage slices were digested with 0.25% trypsin for 30 min at 37°C and they were immersed in 2 mg mL<sup>-1</sup> of collagenase in DMEM supplemented with 5% FBS and incubated overnight at 37°C. Isolated chondrocytes were resuspended by pipetting several times and filtered through a 100 µm filter to remove undigested cartilage fragments. Thereafter, the cell suspension was collected by centrifugation at 1000 rpm for 10 min. The pellet was suspended in complete culture medium (DMEM/Ham's F12+10% FBS). Cell viability was quantified by trypan blue exclusion assay and was constantly 90%.

**Immunocytochemical staining:** Chondrocytes (1.5×10<sup>5</sup> cells/well) were seeded as primary culture on glass coverslips that were coated with collagen type I (0.1 g collagen in 100 mL<sup>-1</sup> acetic acid 0.1%) for immunocytochemistry. Next morning, medium was changed with medium containing 5% FBS and next day, it was changed with medium containing 1% FBS supplemented with BMP-2 (100 ng mL<sup>-1</sup>) for a test group and 1% FBS alone for a control group. The culture medium was replaced every day. After 6 days, indirect immunocytochemical staining was used to detect expression of caspase-3 and performed according to the ABC staining kit (Santa Cruze). The cells were fixed with 4% paraformaldehyde and washed with phosphate buffered saline (PBS) twice. The cells were blocked for 5 min in 0.3% H<sub>2</sub>O<sub>2</sub>, 2 min in 0.2% Triton-X100 and then 1 h in blocking serum. Then, the cells were incubated in primary antibody, a mouse anti-caspase-3 (1:100), for 1 h at 37°C. Positive immunocytochemistry was visualized by avidin-biotin-complex technique according to the ABC kit. Primary antibody was replaced by PBS as a negative control. The percentage positive staining of caspase-3 in

chondrocytes was calculated by counting the number of positively and negatively stained cells. The number of positive chondrocytes was divided by the total number of chondrocytes to calculate the positive chondrocyte ratio.

**MTT-assay for cell number:** Cell proliferation was assessed by a colorimetric methylthiazol tetrazolium (MTT) assay as described by Mosmann (1983), which measured mitochondrial dehydrogenase activity of viable cells spectrophotometrically. OA and normal chondrocytes were cultured in 20 wells of 96-well plates ( $15000 \text{ cells well}^{-1}$ ) and after the cells attached, medium in 8 wells were changed with medium containing 1% FBS supplemented with  $100 \text{ ng mL}^{-1}$  BMP-2, 8 wells changed with medium containing 1% FBS and 4 wells with medium containing 10% FBS. After 6 days, the medium of each well was discarded and  $200 \mu\text{L well}^{-1}$  of the MTT solution ( $0.5 \text{ mg mL}^{-1}$ ) was added and incubated for an additional 3 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Then, the medium was discarded from the wells and added  $200 \mu\text{L well}^{-1}$  of DMSO to dissolve the formazan crystals. The colour intensity was measured at 620 nm using an ELISA reader.

**Statistical analysis:** The data obtained in this study were nonparametric. The statistics significance were analyzed by two-independent samples test and using Mann-Whitney analysis. Values of  $p < 0.05$  were considered significant.

## RESULTS

After histological preparation, the cartilages were evaluated according to the Collins and McElligott (1960) scale for severity of OA. The score in these samples varied from 0 (normal cartilage) to 3 (OA cartilage). In addition, after mechanical and enzymatic isolation of the chondrocytes, we quantified cell rate using trypan blue exclusion assay. The average numbers of normal cells per millilitre were  $3 \times 10^5$  and OA cells were  $2.1 \times 10^5$ . No

significant difference was observed in the average number of OA and normal cells because the weight of normal cartilage was a little higher than the OA cartilage.

**Effect of BMP-2 on morphology:** The morphology of chondrocytes was evaluated by invert microscopy. After mechanical and enzymatic isolation, the freshly isolated normal chondrocytes were polygonal with large, round nuclei. OA cells were smaller in size and nuclei and some of them had clear cytoplasmic and membranous apoptotic changes that they disappeared after the first change of medium. After 6 days, normal chondrocytes in the presence of  $100 \text{ ng mL}^{-1}$  BMP-2 did not show any changes in appearance and they had chondrocyte-like morphology (Fig. 1a). The morphology of OA cells in the presence of BMP-2 was identical to the normal cells but some of them were elongated and showed fibroblast-like morphology after 6 days. The normal and OA cells cultured with 1% FBS alone (control groups) showed round and polygonal shape and a number of dead cells were seen in these groups after 6 days. The normal and OA cells cultured with 10% FBS showed an elongated and fibroblast-like morphology after the same time (Fig. 1b).

**Effect of BMP-2 on proliferation:** The effect of BMP-2 on chondrocyte proliferation in monolayer culture was evaluated with MTT-assay. After 6 days, the normal cells treated with BMP-2 (test group) showed a slow growth rate and mean of absorbance was 0.135. This rate in the normal cells with 1% FBS (control group) approached the treated cultures and mean of absorbance was 0.125. This rate in the OA chondrocytes treated with BMP-2 (test group) was 0.209 and with 1% FBS (control group) was 0.182. The mean absorbance of normal cells with 10% FBS was 0.244. For calculating of percentage of proliferation, the mean absorbance of each group was divided to absorbance of cells with 10% FBS. These percentages were 54 and 51% in test and control groups of normal cells

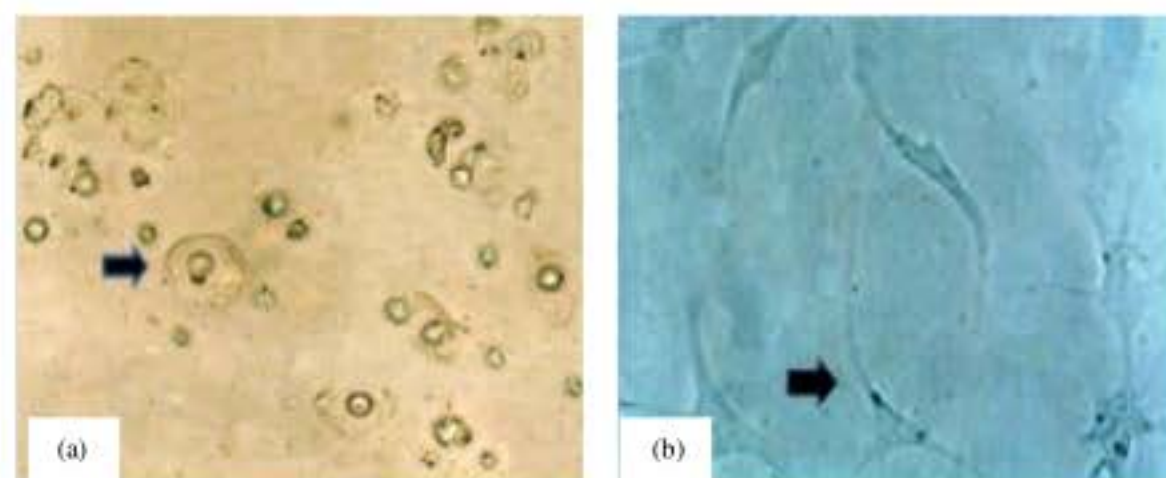


Fig. 1: Morphology of articular chondrocytes derived from human femoral cartilage in the presence of (a)  $100 \text{ ng mL}^{-1}$  BMP-2 and (b) 10% FBS after 6 days. BMP-2 treated cells are round shape (arrow) and cells cultured in 10% FBS exhibited elongated and fibroblast-like morphology (arrow)

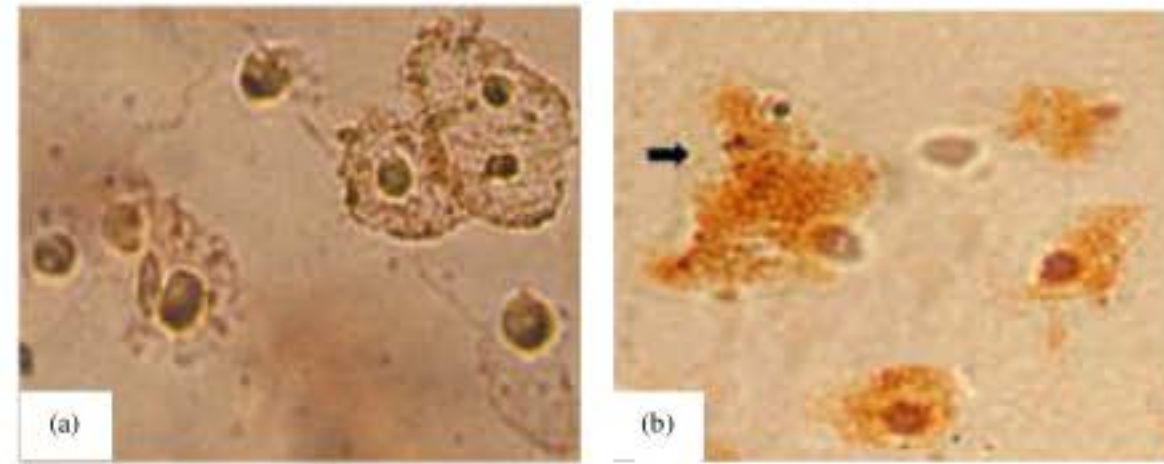


Fig. 2: Caspase-3 positive immunoreactivity of articular chondrocytes derived from human femoral cartilage in the presence of (a) 100 ng mL<sup>-1</sup> BMP-2 and (b) 1% FBS after 6 days. The cells treated with BMP-2 showed no detectable staining. The cells with 1% FBS showed clear and detectable staining (arrows)

Table 1: Effect of BMP-2 on proliferation of normal and osteoarthritic chondrocytes with MTT assay

Groups	Mean of absorbance	Percentage <sup>3</sup>	p-value
10% FBS	0.244		
N <sup>1</sup> BMP-2	0.134	54.0	NS <sup>1</sup> , <0.05 <sup>4</sup>
N 1%FBS	0.120	51.0	NS
OA <sup>2</sup> BMP-2	0.209	85.5	NS, <0.05 <sup>4</sup>
OA 1% FBS	0.182	74.0	NS

NS<sup>1</sup>: Not significant (p>0.05). <sup>4</sup>Significant difference between proliferation rate of normal and OA chondrocytes in presence 100 ng mL<sup>-1</sup> BMP-2 (p<0.05). <sup>3</sup>Compared with 10% FBS

Table 2: Effect of BMP-2 on apoptosis of normal<sup>1</sup> and osteoarthritic<sup>2</sup> chondrocytes with anticaspase-3 immunostaining

Groups	Mean of apoptosis	Percentage	p-value
N <sup>1</sup> BMP-2	5	1	<0.05
N 1%FBS	22	20	<0.05
OA <sup>2</sup> BMP-2	16	30	<0.05
OA 1% FBS	12	43	<0.05

and 85.5 and 74% in test and control groups of OA cells. There was no significant difference between test and control groups but the growth rate in OA test groups were higher than the normal test groups and there was significant difference between OA and normal test groups (p<0.05) (Table 1).

**Effect of BMP-2 on Caspase-3 expression:** To evaluate apoptotic cells, after 6 days, the adherent chondrocytes treated with BMP-2 or 1% FBS alone were stained by anti Caspase-3 and assessed under light microscopy (Fig. 2a, b). In caspase-3 immunostaining, chondrocytes with definite, diffusely stained cytoplasm or nuclei were regarded as positively stained (Fig. 2a) and no staining was detected when anti caspase-3 was omitted from the reaction as a negative control. The mean of apoptotic rates for normal chondrocytes with BMP-2 were 5 (1%) and with 1% FBS were 22 (20%). This rate in OA chondrocytes with BMP-2 were 16 (30%) and with 1% FBS were 12 (43%). The quantitative study indicated a significant difference in the percentage of apoptotic chondrocytes between test groups (culture with BMP-2) and control groups (culture with 1% FBS) and there was

significant difference between normal and OA chondrocytes (Table 2).

## DISCUSSION

The limited capacity of damaged articular cartilage to repair has stimulated many investigations into ways of modulating chondrocyte metabolism. An important strategy to repair cartilage is ACI. Today for improvement of ACI, many studies have done on the cultured chondrocytes. Some of which have involved exploration of the effect of BMPs. In this study, we treated the cultured normal and OA chondrocytes with BMP-2 and compared the characteristics of them.

In current study, when the normal and OA cells were cultured in the presence of BMP-2 in monolayer culture, all of normal cells and the most OA cells were round shape. These results were similar to those have been described in animals (Mallein-Gerin *et al.*, 2003). Brodtkin *et al.* (2004) reported that round cells secreted and organized collagen type II, a behavior consistent with the differentiated phenotype of chondrocytes, but spread cells produces collagen type I and organized fibronectin around the cell, behaviors not consistent with the chondrocytic phenotype. It is well accepted that the round morphology is essential to supporting the chondrocyte phenotype, while flattened fibroblastic-like chondrocytes are dedifferentiated. Treatment of monolayer cultures with BMP-2 modulated the dedifferentiation and kept the chondrocyte morphology and this important feature needs to be considered during the cell amplification procedure for ACI method. In the other hand, Aicher *et al.* (2004) have reported that BMP-2 has no effect on differentiation of human chondrocytes. A possible explanation could be described that chondrocytes were cultured with 10% human serum and medium supplemented with serum accelerate process of dedifferentiation (Stewart *et al.*, 2000). Above

controversy, prompt us to evaluate the morphology and characteristics of human chondrocyte in the presence of 1% FBS.

It is also well known that, the chondrocyte death is a crucial event in the pathogenesis of OA. Many studies have considered whether cell death plays a role in the pathology of OA, because articular chondrocytes cannot self-renew and cell loss would therefore be permanent. However, opinions on the prevalence and importance of chondrocyte death for osteoarthritis pathology differ widely. In this study, we showed that the OA chondrocytes had a higher apoptosis (43%) than the normal chondrocytes (20%) in the presence of 1% FBS. Heraud *et al.* (2000) showed that the apoptotic rate for OA chondrocytes was more important and it was (19-21%) than the normal chondrocytes (4-5%) in monolayer culture. Blanco *et al.* (1998) showed that OA cartilage displayed a higher rate of apoptotic chondrocytes (51%) than normal cartilage (11%) *in vitro*. Kouri *et al.* (1997) showed an apoptotic rate varying from 30 to 88% for four patients with OA. These data are not consistent with our results, because they employed TUNEL assay for the detection of apoptotic cells and this method could be a cause of inconsistency in data recorded. In the other hand, when the OA cells cultured with BMP-2, the rate of apoptosis was dropped (30%). Thus, BMP-2 could reduce apoptosis rate and could be considered as a candidate for treatment of OA disease. In addition, this study showed that BMP-2 could prevent apoptosis rate in the normal chondrocytes and maintain the cell number that it is very important for ACI method.

In addition, in this study we studied the effect of BMP-2 on the proliferation of normal and OA chondrocytes. The present study showed that there is a very low proliferative activity in the normal chondrocyte when they were treated with BMP-2 and this result is in agreement with Richmon *et al.* (2005) that they showed BMP-2 has no effect on expansion of dividing monolayer cultured septal chondrocyte. On the other hand, our study showed that the proliferation of OA cells is higher (74%) than the normal cells (51%) in the presence of 1% FBS. Dozin *et al.* (2002) reported that the cells isolated from OA knee cartilage have limited proliferation capacity. This result possibly could be related to the different sources of chondrocytes. In addition, the proliferation of OA cells had a significant difference with normal cells in the presence of BMP-2. We concluded that the effect of BMP-2 on the proliferation of OA cells is higher than the normal cells.

In conclusion, present study shows that BMP-2 has effect on the apoptosis and dedifferentiation of the normal

chondrocytes higher than the OA cells. So, if we want to use OA cells for ACI method, it may be better to use another growth factor that it can prevent completely apoptosis and dedifferentiation. Finally, further studies are required to clear the other characteristics of BMP-2 and may confirm the hypothesis that this factor could be applied in medical objectives such as ACI or tissue engineering.

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