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RESEARCH ARTICLE

Effect of Chondrogenic Differentiation Medium Supplemented with BMP-9 and TGF-ß3 on Hypertrophy in Transwell Co-Culture

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ABSTRACT

Mesenchymal stem cells are widely used in the treatment of many diseases, including osteoarthritis, due to their ability to differentiate into cartilage. The high chondrogenic differentiation potential of synovial fluid-derived mesenchymal stem cells increases the importance of these cells in osteoarthritis treatments. Addition of BMP-9 and TGF-ß3 into chondrogenic differentiation medium, increases chondrogenic differentiation and they also cause hypertrophic effects on chondrocytes. In our study, it was aimed to demonstrate the effects of BMP-9 and TGF-ß3 on cell hypertrophy by adding them into the chondrogenic basal medium during in vitro chondrogenic differentiation. In the study, stem cells in passage 5 and chondrocytes in passage 1 were cultured in a transwell co-culture system and six experimental groups were formed. Cell hypertrophy was demonstrated by examining MMP-13 and RUNX -2 gene expressions, in stem cells where chondrogenesis were induced in transwell co-culture. Although the addition of BMP-9 and TGF-ß3 to the chondrogenic medium increased hypertrophic gene expressions in experimental groups compared to control, the results were not statistically significant. The addition of BMP-9 and TGF -ß3, separately or in combination, during the chondrogenic differentiation of stem cells does not cause significant chondrocyte hypertrophy.

Keywords: Cartilage, Chondrogenesis, Hypertrophy, Synovial Fluid, Transwell Co-culture

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BMP-9 ve TGF-ß3 Eklenmiş Kondrojenik Farklılaşma Medyumunun Transwell Ko-kültürde Hipertrofi Üzerine Etkisi

ÖZ

Mezenkimal kök hücreler, kıkırdağa farklılaşma yetenekleri nedeniyle osteoartrit dahil birçok hastalığın tedavisinde yaygın olarak kullanılmaktadır. Sinoviyal sıvı kökenli mezenkimal kök hücrelerinin kondrojenik farklılaşma potansiyellerinin fazla olması bu hücrelerin osteoartrit tedavilerindeki önemini artırmaktadır. Kondrojenik farklılaşma medyumuna ilave edilen BMP-9 ve TGF-ß3 büyüme faktörleri kondrojenik farklılaşmayı artırır ve aynı zamanda kondrositlerde hipertofik etkilere sebep olur. Çalışmamızda in vitro kondrojenik farklılaşma esnasında kondrojenik bazal medyuma ilave edilen BMP-9 ve TGF-ß3'ün hücre hipertrofisi üzerine etkilerinin gösterilmesi amaçlandı. Çalışmada transwell ko-kültür sisteminde 5. pasajdaki kök hücreler ve pasaj 1'deki kondrositler birlikte kültüre edildi ve altı deney grubu oluşturuldu. Hücre hipertrofisi, transwell ko-kültüründe kondrogenezin indüklendiği kök hücrelerde MMP-13 ve RUNX-2 gen ekspresyonları incelenerek gösterildi. BMP-9 ve TGF-ß3'ün kondrojenik ortama eklenmesi, kontrol grubuna göre deney gruplarında hipertrofik gen ekspresyonlarını artırmasına rağmen bu artış istatistiksel olarak anlamlı değildi. Kök hücrelerin kondrojenik farklılaşması esnasında BMP-9 ve TGF-ß3'ün ayrı ayrı veya birlikte kullanılması önemli derecede kıkırdak hücresi hipertrofisine neden olmamaktadır.

Anahtar Kelimeler: Hipertrofi, Kıkırdak, Kondrogenezis, Sinoviyal Sıvı, Transwell Ko-kültür

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INTRODUCTION

Synovial fluid mesenchymal stem cells (SFMSCs) have gained importance due to their potential for treatment of osteoarthritis in recent years (Sekiya et al. 2021). It is known that SFMSCs have good therapeutic potential due to their immunosupressive functions (Lee et al. 2015). Besides, they have ability to differentiate into adipocytes, osteocytes and chondrocytes (Jones et al. 2008). Regarding chondrogenic potential of mesenchymal stem cells, it has been suggested that SFMSCs are a better choice compared to other stem cell sources such as bone marrow and adipose tissue (Jones et al. 2008, Murata et al. 2014). Chondrogenesis has been triggered by means of transforming growth factor beta (TGF-ß) family proteins including bone morphogenetic proteins (BMPs) (Kurth et al. 2007, Scarfì 2016). It has been reported that TGF-ß2 and TGF-ß3 have better potential to form cartilage than TGF-ß1 (Barry et al. 2001). TGF-ß3 with dexamethasone on chondrogenic medium enhances aggrecan, type II collagen and cartilage oligomeric matrix protein (COMP) gene expressions on chondrogenesis. (Derfoul et al. 2006). Moreover, TGF-ß3 blocks the terminal differentiation and triggers the initial stages of chondrogenesis (Van der Kraan et al. 2009). Kurth et al. (2007) also demonstrated that this growth factor enhances extracellular matrix production of cartilage. However, Kubosch et al. (2016) showed that TGF-ß3 added culture medium also increases hypertrophy in transwell system which includes synovial mesenchymal stem cells and chondrocytes.

BMP-9 has also higher chondrogenic potential compared to BMP-6 and BMP-2 (Cheng et al. 2016). Nonetheless, BMP-9 not only stimulates chondrogenic differentiation but also causes hypertrophy (Blunk et al. 2003). While TGF-ß activates Smad 2/3 pathway, BMP activates Smad 1/5/8 (Massagué and Wotton 2000). BMPs can induce Runt Related Transcription Factor (RUNX)-2 activity, which is a transcription factor of osteogenesis, via that pathway (Tang et al. 2009). Matrix Metalloproteinase-13 (MMP- 13) is considered as a hypertrophy marker since it is related with calcification (D'angelo et al. 2000). Besides, it was showed that in osteoarthritis (OA) cartilage, upregulation of RUNX-2 gene can increase expression of MMP-13 (Wang et al. 2004). In transwell co-culture, where there is no cell-cell contact, it is possible to observe the cellular changes mediated by the paracrine secretions among the cells, which provides an advantage over other culture systems (Renaud and Martinoli 2016). Moreover, it mimics in vivo cartilage environment when chondrocytes and tissues cultured in it (Aung et al. 2011). It was shown that bone marrow mesenchymal stem cells and chondrocytes which were cultured in transwell system with chondrogenic differentiation medium (containing 10 ng/ml TGF-ß3), decreased Collagen X gene expression which is considered as a hypertrophy marker (Ahmed et al. 2007). However, it is unknown whether addition of TGF-ß3 with BMP-9 or BMP -9 alone to differentiation medium in transwell co-culture induce hypertrophy in the cells during chondrogenesis.

In this study, we established a transwell co-culture system which include SFMSCs and bovine chondrocytes (BCs). We cultured SFMSCs and BCs using chondrogenic differentiation medium supplemented with BMP- 9 and TGF-ß3 in transwell co-culture and detected hypertrophic markers of MMP-13 and RUNX-2.

MATERIALS AND METHODS

Cell Culture of SFMSCs

All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-06-22; 27.01.2022).

Bovine joints were brought from the slaughterhouse (n=6) for collecting synovial fluid and articular cartilage. For SFMSCs isolation, approximately 3-5 mL of synovial fluid was collected from the metatarsophalangeal joint. High glucose (hg)-DMEM (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS) (Biowest, South America) was diluted with synovial fluid at a ratio of 1:2 in a 15 mL centrifuge tube. It was centrifuged at 300 g for 10 min. After discarding the supernatant, the cell pellet was suspended with DMEM-hg (Sigma-Aldrich, USA) containing 10% FBS (Biowest, South America), 1% L- Glutamin (Gibco, UK), 1% penicillin - streptomycin (Gibco, UK), 0.1% amphotericin B (Biochrom, Germany) and cultured in an incubator with 5% CO_2 at 37 ℃ in a 25 cm^2 culture flask. After reaching 70-80% confluence, the cells were passaged. In the 4th passage, the cells were seeded in 6-well plates for transwell co-culture.

Cell Culture of BCs

BCs were obtained from the metatarsophalangeal joint of bovine (n=6). The cartilage was cut with a scalpel blade and put into phosphate buffer saline with 1% pen-streptomycin. For enzymatic tissue digestion, minced cartilage pieces incubated in hg-DMEM containing 0.3% collagenase type II (Sigma,USA) for 25 minutes and then in hg-DMEM containing 0.06 % collagenase type II for 24 hours at 37℃. On the following day, chondrocytes were filtered through a 70 µm cell strainer and centrifuged at 400 g for 10 min (Bernstein et al 2009). Subsequently, cell pellet was suspended with hg-DMEM containing 10% FBS, 0.1% amphotericin B, 1% penicillin -streptomycin and seeded into 25cm^2 flasks. As soon as reaching 70-80 % confluence, the cells were used for transwell co-culture.

Use of BMP-9 and TGF-ß3 with Transwell Co-culture

For chondrogenic induction, 6 groups were planned and shown in table 1. While SFMSCs were used at passage (P) 4, chondrocytes were used at P1. SFMSCs were seeded as 15.000 cells per cm^2 into the surface of 6-well plate then chondrocytes were placed as 15.000 cells per cm^2 at the top the wells using 0.4 μm transwell inserts (Millipore, USA). As SFMSCs basal medium, hg-DMEM with 10% FBS, 1% penicillin - streptomycin and 0.1% amphotericin B was used. For basal chondrogenic medium, hg-DMEM consisting of 5% FBS, 0.1 mM dexamethasone (Sigma, Belgium), 50 mM L-ascorbic acid (Dr. Ehrenstorfer GMbH, Germany), 1% ITS-Premix (Gibco, USA), 1 mM sodium pyruvate (Lonza, Belgium), 0.35 mM proline, 1% non -essential amino acid (Lonza, Belgium) and 1% penicillin-streptomycin (Gibco, UK) was prepared. Chondrogenic differentiation was performed for 21 days according to groups given in table 1. Negative control group (1st group) and 3rd group were cultured with SFMSCs basal medium and other groups were cultured with basal chondrogenic medium supplemented with TGF-ß3, BMP-9 or both. Medium was changed every 3 days. Chondrogenic differentiation protocol was modified from the method of Mackay et al (1998).

Real Time PCR

After culturing BCs together with SFMSCs in the chondrogenic differentiation medium for 21 days in transwell coculture, the cells were collected and stored at -80℃ for real time PCR analysis. A commercial RNA isolation kit (TRIzol Reagent, Thermo, USA) was used for extracting total RNA by following the instructions of the manufacturer and the amount of total RNA was measured with nanodrop. Afterwards, cDNA synthesis was performed according to the kit's instructions (A.B.T., Turkey). 2x qPCR SYBR Green Master Mix kit (A.B.T., Turkey) was used for Real-Time PCR analysis. RUNX-2 and MMP-13 were demonstrated as PCR markers, and GAPDH was included as the housekeeping gene (Table 2).

Statistics

$2^{-\Delta\Delta ct}$ method was used for analysis of real time PCR results and statistics were performed with SPSS 22 software. Shapiro-Wilk univariate normality test was used in order to evaluate the normality of the data. Due to the absence of normal distribution, a non parametric testing was performed using Kruskal-Wallis tests. Data were presented as mean and standard error values in table 3. Kruskal-Wallis test was used to perform multiple comparisons between the groups. Groups were compared with first group which is negative control (Table 3). Statistical significance was determined as $p \leq 0.05$.

Table 1. Experimental Groups

Groups
1st Group: SFMSCs+ SFMSC Basal Medium
2nd Group: SFMSCs+ Chondrogenic Basal Medium with 10ng/ml TGF-ß3
3rd Group: SFMSCs+ BCs+ SFMSC Basal Medium in Transwell System
4th Group: SFMSCs+ BCs+ Chondrogenic Basal Medium with 10ng/ml TGF-ß3 Transwell System
5th Group: SFMSC+ BCs+ Chondrogenic Basal Medium with 10ng/ml BMP-9 in Transwell System
6th Group: SFMSC+ BCs+ Chondrogenic Basal Medium with 10ng/ml BMP-9 and 10ng/ml TGF-ß3 in Transwell System

Table 2. Real Time PCR Primers

Genes	Forward	Reverse	Tm
RUNX-2	CCGGCAGTCGGCTTCRTCGA	AGGGTGGAAATGAGGGGCGA	64 °C
MMP-13	GACCCAGGAGCACTCATGTT	GGTCTTCATCTCCTGGACCATA	64 °C
GAPDH	TGGGCAAGGTCATCCCTGAGC	TCCACAACAGACACGTTGGGA	64 °C

Table 3. Demonstration of MMP-13 and RUNX2 gene expressions. **MMP-13:** Matrix Metalloproteinase-13, **RUNX-2:** Runt Related Transcription Factor-2 **n:** sample size, **x:** mean, **Sx:** standard error, **p:** significance, **ns:** not significant

Groups	n	$\bar{x} \pm S\bar{x}$
1st Group RUNX-2	6	1.00
2nd Group RUNX-2	6	2.10±1.48
3rd Group RUNX-2	6	2.27±1.40
4th Group RUNX-2	6	3.11±0.84
5th Group RUNX-2	6	2.25±0.40
6th Group RUNX-2	6	4.22±1.21
P		**NS**
1st Group MMP-13	6	1.00
2nd Group MMP-13	6	2.10±0.67
3rd Group MMP-13	6	3.40±2.62
4th Group MMP-13	6	3.55±0.80
5th Group MMP-13	6	2.74±1.00
6th Group MMP-13	6	3.70±1.05
P		**NS**

RESULTS AND DISCUSSION

It is known that co -culture system is superior for chondrogenesis than monolayer culture (Xu et al. 2018). Kubosch et al. 2016 indicated that paracrine effects of chondrocytes increased chondrogenic induction of synovial membrane derived stem cells in transwell culture. In this study, we also tried to establish an in vitro transwell co -culture system by combining BCs and SFMSCs to better mimic the environment of natural articular cartilage.

In the beginning of chondrogenic differentiation of the stem cells, Smad 2/3 and Smad 1/5/8 phosphorylation are necessary. Whereas TGF-ß activates Smad 2/3 phosphorylation and blocks terminal differentiation, BMP activates Smad 1/5/8 phosphorylation and triggers chondrogenic hypertrophy (López-Ruiz et al. 2018). TGF-ß3 and BMP-9 are added into condrogenic differentiation medium to differentiate stem cells into chondrocytes TGF- ß3 at a concentration of 10ng/ml has been widely used for chondrogenesis in both co-culture systems (Qing et al. 2011, Wu et al. 2012) and monolayer culture (Sekiya et al. 2012). Kubosch et al. 2016) showed that transwell culture which includes chondrocytes and synovial mesenchymal stem cells treated with 10 ng/ml TGF-ß3 into culture medium increased Collagen X(COL X) expression which is a hypertrophy gene whereas Ahmed et al. (2007) indicated that bone marrow mesenchymal stem cells and chondrocytes which were cultured in same culture system with same amount of TGF -ß3 decreased COL X gene expression. In our study, we also observed that compared to negative group, TGB-ß3 upregulated both RUNX2 (2.10±1.48) and MMP-13 (2.10±0.67) expressions. Besides, compared to group 2, in transwell co-culture of synovial fluid derived stem cells with chondrocytes, TGB-ß3 also increased RUNX2 (3.11±0.84) and MMP-13 (3.55±0.80) expressions more in group 4. In other words, the paracrine effect of chondrocytes increased the chondrogenic hypertrophy of SFMSCs in transwell culture. But the statistically significance was not seen between these groups (table 3).

BMP-9 has high chondrogenic potential on alginate and pellet culture when used at a concentration of 100 ng/ml (Majumdar et al. 2001, Morgan et al. 2020). However, it has been showed that 10 ng/mL BMP-9 possess higher chondrogenic potential than 100ng/ml BMP-9 on C3H10T1/2 mesenchymal stem cells in micro mass culture (Cheng et al. 2016). Also, the hypertrophic potential of BMP- 9 on chondrogenesis has been lesser investigated. In the study of Morgan et al. (2020), it was revealed that the doses of 50, 100 and 200 ng/ml BMP-9 were highly effective in chondrogenic differentiation of chondroprogenitor cells but that doses also caused high hypertrophy. Also, it was determined that the doses of 6, 12.5 and 25ng/ml showed to lead neither high chondrogenic differentiation nor high hypertrophy in that study. In another study, it was showed that 25ng/ml BMP -9 had a hypertrophic effect inactivated by 0.1 ng/ml TGF-ß1 in chondrocytes.

In our study, the highest hypertrophy was observed in 6th group for both MMP-13 (3.70±1.05) and RUNX-2 (4.22±1.21) mRNA levels and the lowest was seen in the 2nd group for both MMP -13 (2.10±0.67) and RUNX-2 (2.10±1.48) compared to negative group. The 5th group which was supplemented with 10ng/ml BMP-9 was observed to have higher gene expressions rates than 2nd group for MMP-13 (2.74±1.00) and RUNX-2 (2.25±0.40) gene expressions. In addition, the 4th group which was supplemented with 10ng/ml TGF -ß3 also showed more hypertrophic condition with higher MMP-13 (3.55±0.80) and RUNX-2 (3.11±0.84) gene expressions than the 5th group. With these results, we could suggest that 10ng/ml TGF-ß3 is more effective than 10 ng/ml BMP-9 to induce hypertrophy in transwell co-culture. When we compare the 3rd group with the 4th group, we think that addition of TGF-ß3 to transwell culture may have been triggered hypertrophy as Kubosch et al. (2016) stated. Even if we see an increase in the gene expressions indicating the cell hypertrophy from data, compared with negative group the observed the increase in the experimental groups was not statistically significant ($p>0.05$) (Table 3).

CONCLUSION

In conclusion, the hypertrophic effect of these doses of BMP-9 and TGF-ß3 during chondrogenesis is limited. The fact that paracrine effects of chondrocytes with these growth factors can induce chondrogenesis with minimal hypertrophy indicates that they are potential candidates for chondrogenesis. In addition, the investigation of the possible hypertrophic effects of different doses of these growth factors at distinct time periods of cell culture during chondrogenesis will be significant for future researches.

Conflict of interest: The authors declared that there is no conflict of interest.

Authors Contribution Rate: 1EEA: %50, ED: %20, ÖÖA: %15, ME: %10, KA:%5

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