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Prostaglandin E2 and hyaluronic acid facilitates treatment of osteochondral deffects

Prostaglandin E2 ve hyaluronik asit sıçan dizinde oluşturulan osteokondral defektlerin onarımında etkilidir

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Objectives

Restoration of the articular hyaline cartilage is the basic prerequisite to preserve normal function of the synovial joint after injury. We have assumed combining hyaluronic acid with prostaglandin E2 (PGE2) will enhance hyaline cartilage formation by stimulating mesenchymal stem cells on fibrin clot scaffold.

Material and methods

Rat knee joint osteochondral defects were treated by (a) bone marrow on fibrin clot, (b) hyaluronic acid alone, (c) bone marrow on fibrin clot combined with PGE2, (d) bone marrow on fibrin clot combined with hyaluronic acid, (e) bone marrow on fibrin clot with hyaluronic acid and PGE2, and (f) a control group left without treatment.

Results

Evaluations were carried out at week six in all groups by radiology and histology. Coverage of defective area with various grades of fibrous or hyaline cartilage was noticed in all groups. All treatment groups presented better healing patterns than the control group. Hyaline cartilage with normal matrix staining pattern was observed on Safranin-O stained sections from (1) bone marrow on fibrin clot with PGE2 and (2) bone marrow on fibrin clot with hyaluronic acid and PGE2. Healing response at these groups was statistically better then the control group.

Conclusion

This study demonstrates beneficial effects of PGE2 and hyaluronic acid during chondrocyte differentiation. These properties of PGE2 and hyaluronic acid will have a role during the development of new strategies in osteochondral defect treatment.

Key words: PGE2, Hyaluronic acid, Cartilage healing, Bone marrow, Fibrin clot

Amaç

Hyalin eklem kıkırdağının korunması ve onarımı eklem işlevinin korunabilmesi açısından gereklidir. Bu çalışmada hyaluronik asit ve Prostaglandin E2' nin (PGE2) birlikte kullanılmasının fibrin pıhtı içerine emdirilmiş kemik iliği hücrelerinden hyaline kıkırdak dokusunun gelişmesini uyarabileceği varsayımını araştırdık.

Gereç ve yöntemler

Sıçan dizlerinde oluşturulan osteokondral lezyonlar belirlen çalışma protokollerine uygun olarak tedavi edildi. Buna göre gruplar; (1) kemik iliği ve fibrin pıhtı karışımı, (2) sadece hyaluronik asit, (3) fibrin pıhtı, kemik iliği ve PGE2, (4) fibrin pıhtı, kemik iliği ve hyaluronik asit (5) fibrin pıhtı, kemik iliği karışımı ile hyaluronik asit PGE2 karışımı, (6) tedavi verilmeyen kontrol grubu olarak belirlendi. Altıncı haftanın sonunda çalışma sonuçları histolojik olarak değerlendirildi.

Sonuçlar

Tüm tedavi gruplarında kontrol grubundan belirgin olarak farklı derecelerde fibröz ve hyaline kıkırdak iyileşmesi saptandı. Fibrin pıhtı, kemik iliği ve hyaluronik asit karışımın kullanıldığı grup ile fibrin pıhtı, kemik iliği karışımı ile hyaluronik asit ve PGE2'nin birlikte kullanıldığı gruplarda Safranin-O ile boyanmış kesitlerde normal hyalin kıkırdak ağ yapısı ile aynı boyama özellikleri gösteren kıkırdak matriksin oluştuğu görüldü. Bu bulgu konrol grubundan anlamlı ($p<0.05$) farklıydı.

Çıkarım

Bu çalışmanın sonuçları, PGE2 ve hyaluronik asit'in birlikte kullanılmasının kıkırdak onarımı ve farklanmasını olumlu etkilediğini göstermiştir. PGE2 ve hyaluronik asit'in bu özellikleri osteokondral lezyonlarının tedavisinde yeni stratejilerin belirlenmesinde yararlı olacaktır. Ayrıca, uygun ortopedik tedavinin yanında akut osteokondral yaralanmalarda eklem içine hyaluronik asit enjeksiyonlarıda klinik uygulamada yararlı olabilir.

Anahtar sözcükler: PGE2, Hyaluronik asit, Kıkırdak iyileşmesi, Kemik iliği, Fibrin pıhtı

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Loss of hyaline cartilage integrity may result in subsequent joint degeneration and may become the source of pain and loss of function.[1-3] Restoration of hyaline cartilage in injured and/or degenerated synovial joints is the basic prerequisites for preservation of normal function.[2] Cost of treatment of joint degeneration is high[1], and clinical and socio-economical results are not always promising.

Grafting with osteochondral units is a surgical treatment option for focal articular cartilage lesion. Despite encouraging results were reported development of donor site morbidity, amount of available material, difficulty to restore the normal topology and loss of cells and matrix components at the transplant borders are main points of concern.[1, 2, 4, 5]

Stimulation of chondrocytes and matrix formation by promoting migration of new progenitor cells into defective area by means of increasing blood flow or stimulation of intramedullary mesenchymal multipotention cells through the breakthrough of the subchondral bone plate is an philosophy for restoration of joint hyaline cartilage.[2] Autogenous bone marrow mesenchymal stem cells can be stimulated by biologically active substances such as; BMP-2, IGF-1, TGF-β.[12, 6-10] Recent in-vitro studies have claimed anabolic effects of PGE2 on chondrogenesis.[11-13] Results of these studies suggest that simultaneous stimulation of EP2 and EP4 subreceptors of PGE2 is necessary to observe this effect. Both EP2 and EP4 subreceptors exist together in mice, rats and humans .[14]

Hyaluronic acid is the simplest gylcosaminolglycan and it is mainly found in cartilage tissue. It has diverse functions; it acts as a mediator during tissue development and can be used as a scaffold for cartilage engineering.[1, 15, 16] Fibrin polymer is another natural, stable structure which facilitates the growth of collagen producing fibroblasts, delivers the growth factors and emulates the exudative phase of wound healing.[17]

We assumed that; combination of hyaluronic acid together with PGE2 will enhance hyaline cartilage formation by stimulating bone marrow mesenchymal stem cells on a fibrin polymer scaffold. Evaluations were carried out at week six in all groups by histology.

MATERIALS AND METHODS

Animals and Surgery

Thirty skeletally mature (6 months of age) male Wistar rats weighing approximately 250gr were obtained from Refik Saydam Hifzissihha Institute (Ankara–Turkey). The left knee was exposed by an anterior parapatellar midline incision. A cylindrical full thickness osteochondral defect of a diameter of 1.5 mm with a depth of 1.5 mm was created by a drill at the anterior surface of the patella-femoral groove. Free movement was allowed after surgery. All surgical procedures were accomplished after intramuscular administration of 60 mg/kg ketamine (Ketalar, Pfizer Inc, USA) and 10mg/kg xylazine (Alfasan International, Woerden, Holland). Paracetamol for a day was given to decrease pain after surgery.[12] Animals were terminated using high dose of thiopental (I.E. Ulagay Pharmaceuticals, Istanbul - Turkey). All procedures were in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26 and with the Helsinki Declaration of Animal Rights. Study protocol was approved by the Ethical Committee of Ankara Numune and Research Hospital on 07.12.2005 by decision code 2005/129. Following termination at sixth week, left lower extremities of rats were surgically removed. All extremities underwent histological examination.

Groups

Five rats were allocated to each group. Group 1 was assigned as the control with no treatment for osteochondral defects. Defects at Group 2 were filled with bone marrow on fibrin clot. Group 3 received bone marrow on fibrin clot combined with 10-7 mMol PGE2 (Acros, Belgium). Defects of Group 4 were filled with 0,1 cc high molecular weight (500,000-730,000 daltons) hyaluronic acid (Hyalgan - Fidia, Italy). Defects of Group 5 rats were filled with bone marrow on fibrin clot combined with 0.1 cc hyaluronic acid. Defects of Group 6 were filled with bone marrow on fibrin clot combined with 10-7 mMol PGE2 and 0.1ml hyaluronic acid. Intraarticular hyaluronic acid injections were continued for three consecutive weeks in all hyaluronic acid applied groups (Groups 4, 5 and 6). Rats did not receive any other form of treatment.

Preparation of Fibrin Clot and Bone Marrow: Two skeletally mature rats were terminated by hypovolemic shock. Collected blood samples from intracardiac aspiration were passed into tubes containing 3.2 % sodium citrate. These blood samples were than centrifuged for 3 minutes at 5000/rpm. Serums were than mixed with 10 ml of 10% calcium levulinate (Adeka Pharmacetucals, Samsun-Turkey). This mixture was than shaken for 5 minutes to facilitate fibrin clot formation. Formed clot was frozen to -18°C. Frozen mixture was than thawed to room temperature and precipitated clot was separated under sterile conditions.[18, 19] Bone marrow aspirates were also prepared from the tibia and femurs of sacrificed animals.

Histopathology

Samples were fixed in 10% phosphate-buffered formalin (pH= 7.0). Decalcification process accomplished with De Castro solution (30 ml, 70 % nitric acid, 50 g Chloral Hydrate, 300 ml ethanol) at room temperature. Afterwards, specimens were dehydrated in increasing degrees of ethanol and

embedded into paraffin blocks under fixed vacuum. Six to eight µm thick serial transverse sections were fixed to gelatin coated lams by using a rotary microtome (Microm, HM 360, Germany). In order to catch cartilage defects, 2-3 transverse sections were omitted. Specimens were stained with Hemotoxylin and Eosin and Safranin-O. All sections were evaluated with a Leica DMR light microscope. Images were captured with a DC 500 model Leica digital camera. Samples were evaluated for the morphology of repair tissue, Safranin-O staining of new matrix, smoothness of joint surface at defect area, integration of new matrix with surrounding tissues, amount of regenerated tissue at defect site and presence of degenerative changes within defective area. For this purpose, modification of a semi-quantative scoring scale was used.[5, 20] Each section was evaluated by two independent histologists and their average was taken for statistical evaluation.

Statistical Evaluation: Histological scores were statistically analyzed with Kruskal-Wallis test and Mann-Whitney U test. In order to eliminate problems originating from small sample size of study groups, statistical analyzes were performed with Bonferroni correction. Statistical calculations were performed with the SPSS 13.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Various stages of cartilage healing was accompanying the regeneration and restructuring of bone underlying the defects at all treatment and control groups at the end of six weeks. Coverage of defective area with various grades of fibrous or hyaline cartilage was noticed in all groups (Figure 1-A, 1-B). All treatment groups presented better healing patterns than the control group.

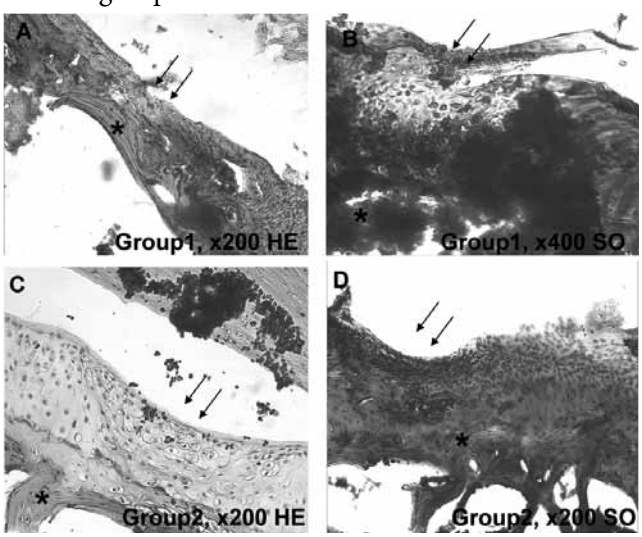

Figure 1. (A-B): Micrographs from group 1 (Control group) and (C-D) Group 2 (bone marrow on fibrin clot). Osteochondral defect filled with fibrous cartilage tissue is remarked as areas of weakly patchy staining with Safranin-O (arrows). The defect area has an uneven surface with reformation of subchondral bone (*). (HE: Hematoxylene-Eosin, SO: Safranin-O)

In Group 2 (bone marrow on fibrin clot), defect was filled with fibrous cartilage islets which was accompanied by disorganized chondrocyte clusters. Joint surface was uneven at this region (Figure 1-C, 1-D). Fibrin was entirely replaced with cartilage. There were patchy hyaline cartilage areas and newly formed cartilage areas which were in continuity with surrounding cartilage tissue. Hyaline cartilage formation was better in only hyaluronic acid applied group (Group 4) compared to Group 2 (Figure 2-C, 2-D).

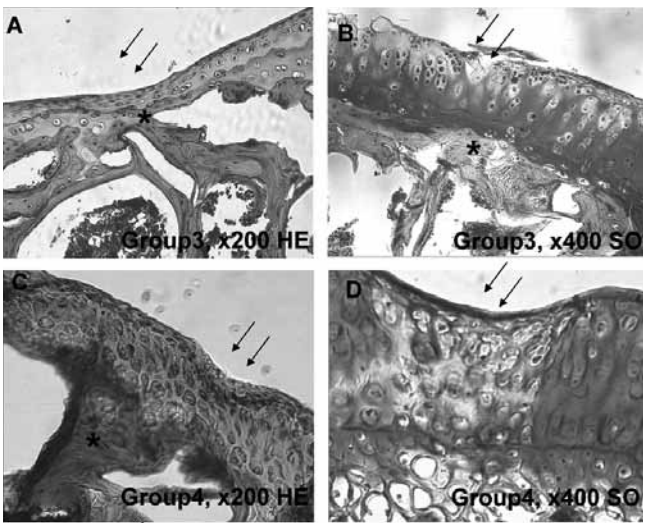

Figure 2. (A-B) Micrographs from Group 3 (bone marrow on fibrin clot combined with PGE2) and (C-D) Group 4 (hyaluronic acid). Hyaline cartilage in defect area appeared red with Safranin-O (arrows). Even though the defect surface is minimally depressed, subchondral bone started to reform itself (*). (HE: Hematoxylene-Eosin, SO: Safranin-O)

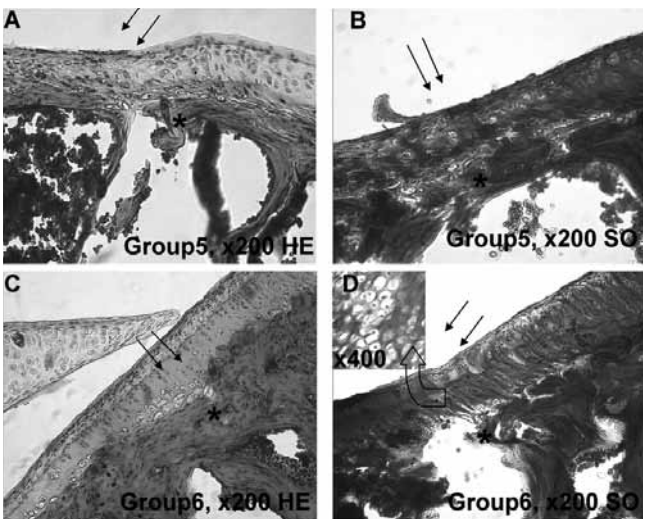

Figure 3. (A-B) Micrographs from Group 5 (bone marrow on fibrin clot combined with hyaluronic acid), and (C-D) Group 6 (bone marrow on fibrin clot combined with PGE2 and hyaluronic acid). The hyaline cartilage appears almost normal in defect area with its red color in Safranin-O (arrows). On sections from Group 6, defect surface gained an even appearance, making it difficult to differentiate from surrounding tissues. Magnified insert on section D, shows reformed chondrocyte columns. (HE: Hematoxylene-Eosin, SO: Safranin-O)

However, there was no significant difference between these two treatment groups (Figure 3). Hyaline cartilage with normal matrix staining pattern was observed by Safranin-O staining in specimens of Group 5 (bone marrow on fibrin clot and hyaluronic acid), (Figure 3-A, 3-B) and Group 3 (bone marrow on fibrin clot with PGE2) (Figure 2-A, 2-B). However, chondrocyte clusters were partially dispersed. Even though joint surface was fairly smooth, it was partially collapsed as the outer layer of cartilage was not well formed. Subchondral bone healed to its normal architecture. Figure 3 (A-B): Micrographs from Group 5 (bone marrow on fibrin clot combined with hyaluronic acid), and (C-D) Group 6 (bone marrow on fibrin clot combined with PGE2 and hyaluronic acid). The hyaline cartilage appears almost normal in defect area with its red color in Safranin-O (arrows). On sections from Group 6, defect surface gained an even appearance, making it difficult to differentiate from surrounding tissues. Magnified insert on section D, shows reformed chondrocyte columns. (HE: Hematoxylene-Eosin, SO: Safranin-O)

Fastest healing was observed in Group 5 (bone marrow on fibrin clot with hyaluronic acid) (Figure 3-A, 3-B) and Group 6 (bone marrow on fibrin clot with PGE2 and hyaluronic acid) (Figure 3-C, 3-D). Newly formed tissue presented healthy hyaline cartilage properties. Extracellular matrix was homogenously stained red with Safranin-O, and cartilage tissue healing was in continuity with surrounding tissues. Subchondral bone also developed well. Healing response at these groups was statistically ($p<0.05$) better then Group 1 (control) and Group 2 (bone marrow on fibrin clot)(Figure 4)(Table 1).

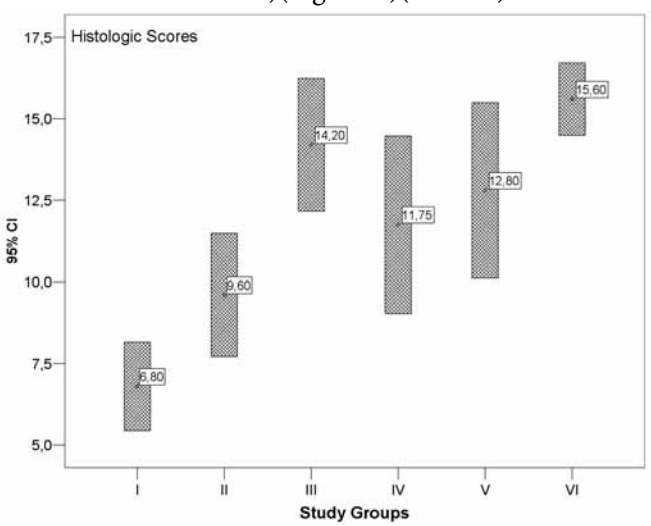

Figure 4. Mean scores of histologic evaluation at 95% confidence interval for mean. [Group 1 (control group), Group2 (bone marrow on fibrin clot), Group 3 (bone marrow on fibrin clot combined with PGE2), Group 4 (hyaluronic acid), Group 5 (bone marrow on fibrin clot combined with hyaluronic acid), Group 6 (bone marrow on fibrin clot combined with PGE2 and hyaluronic acid)]

Table I.
Summary of histological scoring.

Group	Number of Rats	Median	Std. Deviation	Mean	Minimum	Maximum
1.00	5	9,2000	1,48324	9,0000	7,00	11,00
2,00	5	12,4000	2,07364	13,0000	9,00	14,00
3,00	5	16,2000	4,76445	16,0000	9,00	21,00
4,00	4	16,0000	3,55903	15,0000	13,00	21,00
5,00	5	17,4000	3,50714	16,0000	13,00	21,00
6,00	5	19,6000	2,60768	21,0000	15,00	21,00

DISCUSSION

During this study, our aim was to engineer a composite material which can promote restoration of damaged or lost hyaline articular cartilage. We used the bone marrow as the cell source and demonstrated the additive effects of PGE2 and hyaluronic acid on stimulation of chondrogenesis. Histological evaluations of treatment groups showed various stages of reformation of hyaline cartilage and subchondral bone architecture in the treatment groups. Defective areas were covered with various grades of fibrous or hyaline cartilage. Healthy hyaline cartilage reformation was most prominent in Group 6 (PGE2 and hyaluronic acid combination). This was significantly different than Group 1 (control) and Group 2 (bone marrow on fibrin clot). There was no sign of healing at the control group at histological evaluations.

Adult articular cartilage has a limited healing potential1. Complete reformation of hyaline cartilage surface was rarely reported in previous in-vitro and in-vivo studies, which is evenly in continuity with the surrounding joint surface.[1, 21] This is mostly because of absence of vascularization within the cartilage tissue and limitations of chondrocytes to dedifferentiate for subsequent regeneration.[2, 3] This in turn severely affects the healing capacity of articular cartilage.

Hyaluronic acid is the simplest gylcosaminolglycan and it is mainly found in cartilage tissue. It has diverse functions; it acts as a mediator of tissue development and cell differentiation and also posse's important functions in fluid homeostasis. Through this actions, hyaluronan plays an important role for the functional and structural integrity of cartilage tissue1. Hyaluronan has received attention as a scaffold material for cartilage tissue engineering. One special feature of this polysaccharide is its high turnover rate in the tissue. Hyaluronan is degraded by locally secreted enzymes, named hyaluronidases, allowing tissue turnover by cells in the skeletal defect site. Cellular effects of exogenous hyaluronic acid include increasing endogenous hyaluronic acid synthesis, stimulation of proteoglycan synthesis and inhibition of degrading

enzymes within the joint. They inhibit mononuclear cell phagocytosis, leukocyte migration, chemotaxis and act as free radical scavengers.[1, 15, 16]

In addition to these properties they have been shown to increase survival of implanted cells by providing a better environment for diffusion when they are added to cell mixtures. We observed cartilage healing on only hyaluronic acid applied groups that may be due to the above mentioned properties of hyaluronic acid on cells coming through the breakthrough of the subchondral bone plate.

Scaffolds provide a structural basis for cells to form a three dimensional tissue construct and determine the mass transport.[1] There are two types of scaffold materials that can be used for cartilage regeneration; (1) synthetic organic materials which are biodegradable polymers like polygylcolide, polylactides, polydioxanone and polycaprolactone and (2) natural organic materials such as collagen, fibrin and hyaluronic acid. Synthetic biodegradable materials are prone to develop foreign body reactions and their degradation products may also be another source of local problems. Therefore; natural organic materials may be a better alternative as a cartilage scaffold.[1] Fibrin clot may provide advantages over other natural materials. Fibrin clot works as an adhesive by emulating the exudative phase of wound healing. As a stable polymer, fibrin clot facilities the growth of collagen producing fibroblasts and provides a suitable environment for delivery of local substrates.[17] We think combination of hyaluronic acid with fibrin clot yields into a more favorable environment during early phases of regeneration.

Bone marrow stem cells were shown to promote cartilage healing in previous studies 2. Biologically active substances such as BMP-2, IGF-1, TGFβ-1 and PGE2 have stimulatory effects on bone marrow mesenchymal stem cells to differentiate into condrocytes.[11, 13]

Prostaglandins play an important role during various physiological and pathological processes and they are produced from arachidonic acid. Their role on cartilage metabolism is controversial, however, their stimulatory effects on chondrogenesis and terminal differentiation of chondrocytes has been presented.[3, 6,11-13] PGE2 exert its functions through EP receptors. There are four subgroups of EP receptors. Previous studies have shown that PGE2's effects on chondrocyte differentiation are through EP2 and EP4 receptors.[7-10] Type of sub-receptors on which they act and milieu of tissue applied effects the outcome. Previous studies have reported their stimulatory effects on bone marrow cells through similar receptors.[22] Results of the current study demonstrate that they may also have a beneficiary role on cartilage formation. Addition of hyaluronic acid to composite enhances this activity. Both EP2 and EP4 sub-receptors of PGE2 only exist together in mice, rat and humans.[14] This was the reason why we preferred the rat knee joint model in this study.

There are three basic methods to evaluate the outcome of cartilage defect healing in animal models. These are (1) histology, (2) biochemistry and (3) biomechanical testing. Unfortunately none of them can successfully be used in the clinical setting. In patients, pain relief and a good function can be assessed only. In experimental setting, histology provides the broadest range of information and is widely accepted. Mechanical and biochemical evaluations are more appropriate for more specialized follow-up studies.[23] Histological methods allows the evaluation of many important factors in the reparative process like types of tissues filling the defect (including both cell and extracellular matrix (ECM) characteristics), attachment to adjacent structures (cartilage, calcified cartilage or bone) and the health of the adjacent tissues.[23] Safranin-O is a cationic dye that binds to polyanions but not to collagen. According to Gruber and Ingram, Safranin-O staining offers more reliable information on articular cartilage repair.[24]

Lack of healing at the femoral osteochondral defect site at six weeks in the control group was obvious. Rat cartilage has a high turn-over rate[25], which makes it possible to observe the healing response at six weeks. However, small sample size in groups and lack of biomechanical tests were the disadvantage of this model.

We have demonstrated the stimulatory effect of PGE2 on bone marrow cells in chondrocyte differentiation. Adding hyaluronic acid to the composite augmented the positive effects. Hyaluronic acid directly stimulated bone marrow stem cells and provided a better environment for cells in their new locations. Subsequent intra-articular injections facilitated the healing process. To conclude, in addition to known orthopedic approaches, the effect of PGE2 and hyaluronic acid will have a role during developing new strategies in treatment of osteochondral defects.

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