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Investigating the comparative effect of conditioned medium from mesenchymal stem cells and fibroblast cells on articular cartilage defects

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ABSTRACT: Mesenchymal stem cell-secreted factors play influential roles in proliferation, differentiation, and chondrogenesis. This study was designed to examine the ameliorative effects of conditioned medium (CM) from stem cells, fibroblasts, and platelet rich plasma (PRP) on articular cartilage defects in a rat model. A cartilage defect (2 mm in diameter) was created in the intercondylar notch of the femur. Forty adult male Wistar rats were divided into eight groups: intact, sham, and untreated, DMEM, stem cells, stem cell conditioned medium, fibroblast conditioned medium, and PRP. Conditioned medium, PRP, and stem cells were administered as a single dose intra-articularly immediately after surgery and closure of the joint capsule. At the end of the 4th week, cartilage repair was assessed by histopathological assays. Microscopy data showed that defects in the stem cell-conditioned medium group repaired tissue better in terms of cartilage layer formation, thickness, collagen deposition and glycosaminoglycan synthesis compared to defects in the stem cell, fibroblast-conditioned medium, and PRP groups. In terms of angiogenesis, the fibroblast-conditioned medium group outperformed the other groups. Based on histological findings, stem cell conditioned medium yields superior results to fibroblast conditioned medium and PRP treatment in creating hyaline-like repair tissue. The effectiveness of the role of conditioned medium compared to cell therapy may only be due to the type and amount of growth factors present in them.

KEYWORDS: Stem cell-conditioned medium; fibroblast-conditioned medium; cartilage defect; cartilage repair.

1. INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis, which affects more than 240 million people worldwide that is defined by articular cartilage damage, subchondral bone sclerosis and osteophytosis. The clinical symptoms of OA are chronic pain during movement and physical disability, which reduces the quality and life expectancy of people. It is estimated that millions of people have symptomatic OA in the knee joint. Age and gender, diet, obesity, metabolic syndrome, and genetic factors are risk factors for OA [1]. Traumatic injuries and many joint diseases, such as OA, can damage the cartilage layer of the joint. Since articular cartilages are without blood vessels and are fed through diffusion, their ability to self-repair is limited. Defects in articular cartilage can also lead to osteoarthritis at a later stage. Current treatment for cartilage defects includes physical therapy, oral medications, and intra-articular injections. Also, chondroplasty is an alternative procedure that can relieve pain. However, these treatments do not play a role in cartilage tissue regeneration, and cartilage defects can progress and lead to more severe joint cartilage damage [2,4].

Cell therapy and implantation of autologous cartilage cells are the gold standard methods for cartilage repair in the clinic. But the use of autologous cartilage cells requires an additional surgery and destruction of a part of a joint and an invasive surgical procedure [5,6]. In recent years, mesenchymal stem cells (MSCs) have been proposed for the treatment of OA and to promote neocartilage formation [2]. Various pathways are involved in the development of cartilage or joint formation, which are also related to the pathogenesis of OA and can theoretically serve as therapeutic targets. Among these signaling factors, the role of fibroblast growth factors (FGFs) by binding to fibroblast growth factors receptor (FGFR) induce tyrosine phosphorylation. FGFRs are expressed in adult human articular chondrocytes. Also, FGFs are secreted proteins that are

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abundantly expressed in during embryonic development. It has also been reported that FGF/FGFR signaling plays a critical role in maintaining the homeostasis of chondrocyte destruction and repair [1].

The effect of conditioned medium (CM) of mesenchymal stem cells on the survival of intervertebral disc cells and the production of extracellular matrix was confirmed by the researchers [7]. CM plays an effective role in the proliferation, migration of chondrocytes, and glycosaminoglycan deposition. CM with paracrine-like activity regenerate and delay the degeneration of osteochondrogenesis injury [8]. One of the advantages of this treatment method compared to cell therapy is ease of preparation and easy transportation. They are also potentially hypoimmunogenic due to being acellular.

So far, the evaluation of the effect of CM derived from fibroblast on articular cartilage defects has not been investigated. The purpose of this study is to determine the effect of CM obtained from the culture of fibroblast s and stem cells derived from bone marrow on knee articular cartilage repair in the rat animal model after the induction of cartilage defects.

2. RESULTS

2.1. Characteristics of BM-MSCs:

The criteria used in this study for the specificity of BM-MSC mesenchymal stem cells were the adhesion of cells to the bottom of the plate during cell culture and the expression of specific surface markers of stem cells. Flow cytometry evaluation showed that these cells had a positive expression of CD44 and CD105 mesenchymal markers. And the non-mesenchymal markers CD34 and CD45 were not expressed in them.

2.2. Histological findings

Figure 1 shows a micrograph of the repair structure of the cartilage defect of the lower end of the femur in the knee joint four weeks after surgery.

Fig. A is related to the control group without defect. Cartilage has a normal tissue structure in terms of chondrocytes, lacunae and ground substance.

In the group whose joint capsule was opened but no defect was created in the joint, rupture and disintegration of the synovial membrane and accumulation of inflammatory cells to a moderate extent were observed in the area of damage to the joint capsule. But the joint surface remained healthy (Fig. B).

The defect in the group that did not receive the treatment was also filled by a fibrous tissue. But there were fewer fibroblasts, especially near the surface of the defect, and larger blood vessels (Fig. C).

The results of histological findings four weeks after surgery showed that the joint defects treated with cell culture medium (DMEM), the damaged area was filled with fibrous tissue that contained fibroblasts and numerous blood vessels and collagen fibers. The damage area remained hollow and no chondrocyte-like oval cells were seen (Fig. D).

The cartilage structure in the group that received bone marrow stem cells, the proliferation of chondroblastic cells and their transformation into single or double chondrocytes in some areas were surrounded by relatively few lacunae. These changes were in the deep part of the damaged area. But in the surface area of the damaged area, there is a small population of cells and a lot of ground substance that has acidophilic properties and seems to indicate the formation of bone blades. The repaired tissue was almost completely integrated with healthy cartilage (Fig. E).

Cartilage tissue structure in the group that received the stem cell conditioned medium, the proliferation of chondroblastic cells was seen in the defect area. Abundant chondrocytes with a large thickness were found in the damaged area, which indicates the formation of cartilage in the damaged area. Cartilaginous cells were hypertrophied in some places. Some of them became chondrocyte that enclosed in lacunae. But cartilage cells do not have an isoform structure (Fig. F).

The tissue structure of the cartilage in the group that received fibroblast cell conditioned medium, the injury area was free of inflammatory cells. Many vascular sections were observed. Collagen and fibroblasts were also observed in the damaged area. In the depth of the damaged zone, chondrification started, so that the chondrocyte cells had certain lacunae. Also, some cartilage cells were slightly hypertrophied (Fig. G).

The tissue structure of the cartilage in the group that received PRP was hypercellular and cell proliferation was seen in the area of damage and the formation of chondrocyte cells resulting from the proliferation and differentiation of chondroblastic cells, which in some places were enclosed in the surrounding lacunae to some extent. Thick connective tissue with connective tissue cells and relatively large blood vessels, which is associated with vascular congestion, were observed. (Fig. H).

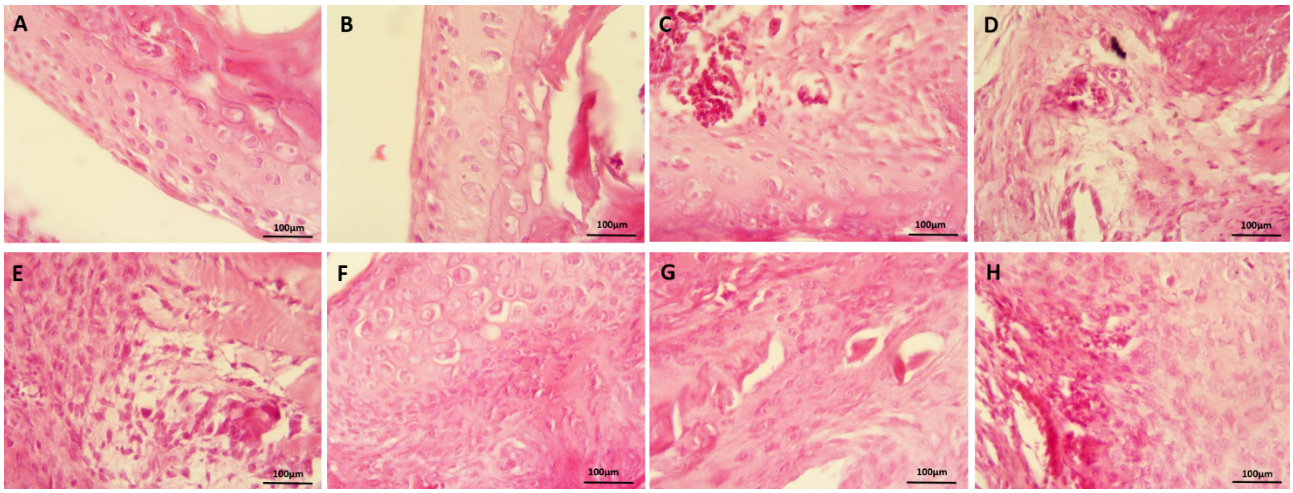

Figure 1. The histological structure of cartilage defect repair in the intercondylar region of the femur shows four weeks after the creation of the defect model in all groups. H&E staining with 40x magnification. A) Control, B) Sham, C) Untreated, D) DMEM, E) Stem cell, F) Stem cell conditioned medium, G) Fibroblast conditioned medium, and H) PRP groups. Mag; 40x, Scale bar; 100µm.

Figure 2 shows photomicrographs from the articular cartilage repair area with Masson's trichrome staining. As shown in Fig. 2, there was a higher repair score in the defects treated with the SCCM, FCM, and PRP compared with DMED and SC groups. These findings demonstrate that SCCM plays a role in cartilage repair.

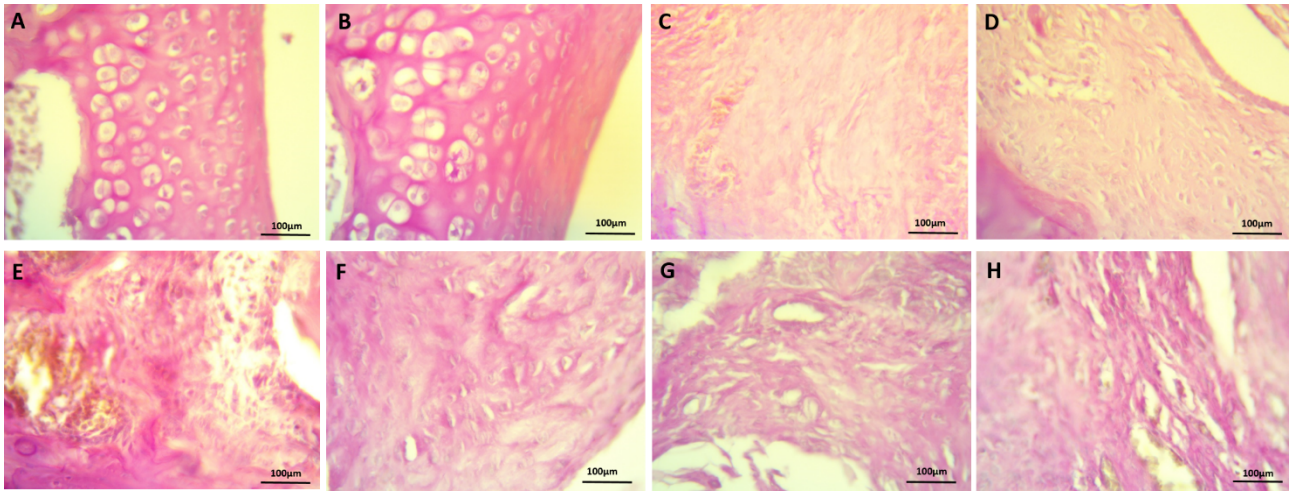

Figure 2. Collagen deposition in the repaired area four weeks after the creation of the defect model in all groups. Masson's trichrome staining with 40x magnification. A) Control, B) Sham, C) Untreated, D) DMEM, E) Stem cell, F) Stem cell conditioned medium, G) Fibroblast conditioned medium, and H) PRP groups. Mag; 40x, Scale bar; 100µm.

Figure 3 shows photomicrographs from the articular cartilage repair area with PAS staining. To investigate glycosaminoglycan levels following cell therapy and Conditioned medium therapy, regenerated tissues were examined using PAS staining. Photomicrographs show that the glycosaminoglycan was deposited in the repaired cartilage in the SCCM, FCM, and PRP groups compared with untreated and DMEM groups (Fig. 3). In particular, in the SCCM, FCM, and PRP groups, the repaired cartilage area has a similar staining design to the surrounding extracellular matrix around the chondrocyte-like cells.

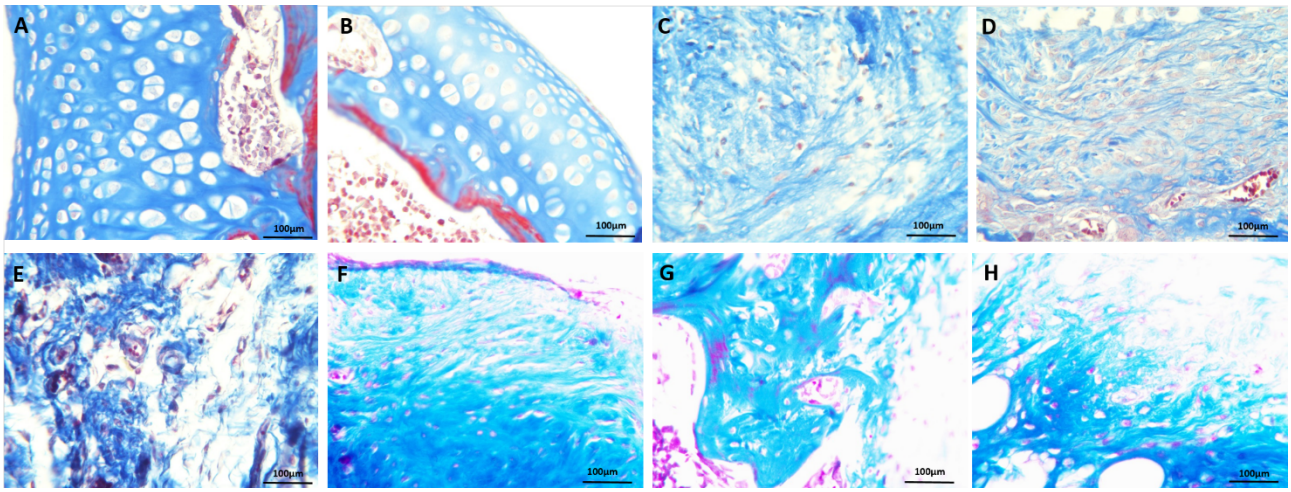

Figure 3. Glycosaminoglycan accumulation in the repaired area four weeks after the creation of the defect model in all groups. Periodic Acid Schiff staining with 40x magnification. A) Control, B) Sham, C) Untreated, D) DMEM, E) Stem cell, F) Stem cell conditioned medium, G) Fibroblast conditioned medium, and H) PRP groups. Mag; 40x, Scale bar; 100µm.

The histological findings were shown in the semi-quantitative evaluation with the modified O'Driscoll histological scoring system in Figure 4. The mean ± standard deviation in the group treated with stem cell conditioned medium, fibroblast conditioned medium and PRP groups were higher than the sham group and treated with DMEM groups, although no statistically significant difference was observed between the two groups of fibroblast and stem cells conditioned medium. These findings indicate that CM and PRP play a role in articular cartilage repair. Meanwhile, SCCM shows a superior role in cartilage repair.

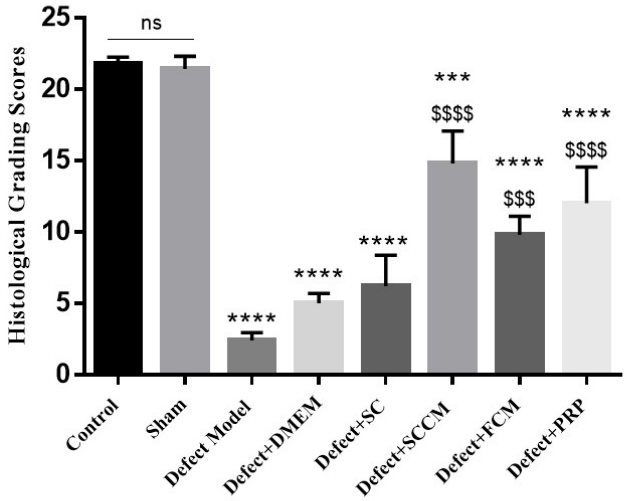

Figure 4. Mean O'Driscoll scoring system for the control and stem cell treated groups at the 4th week after surgery. All data represent the mean ± standard deviation. * indicate statistical differences with the control and $ with defect groups. Abbreviations: CS: Stem Cell, SCCM: Stem Cell Conditioned Medium, FCM: Fibroblast Conditioned Medium, PRP: Platelet rich plasma. "ns" indicates not significant ($p>0.05$).

3. DISCUSSION

This study has successfully demonstrated the possibility of using the conditioned medium of stem cells derived from bone marrow and fibroblast cells as a new and highly convenient strategy in articular cartilage repair. The quality of reconstructed tissue at the defect site treated with stem cell and fibroblast conditioned media was microscopically superior to the treatment with stem cells and culture media

alone. Partial regeneration of articular cartilage was evident. The suitability of PRF in cartilage defect repair was also shown in this study.

In this study, rats were used to create a cartilage defect model. The researchers stated that the PRF of smaller laboratory animals is not of good quality [9], but the results of this study showed that rat PRP was able to play an effective role in the repair of articular cartilage defects.

Growth factors such as TGF-β, fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF) play an effective role in homeostasis and articular cartilage repair [10]. Researchers have shown that FGF1 plays an effective role in the differentiation of human bone marrow mesenchymal stem cells and fracture repair. FGFs can be considered as targets for new therapeutic interventions in bone and cartilage related diseases [11,12]. Furthermore, FGF-2 has also an inhibitory effect on apoptosis, maintains the phenotype of chondrocytes and restores proteoglycan synthesis [13].

The present study showed that platelets had an effective role in cartilage repair. Platelet is a rich source of growth factors (TGF, IGF, FGF) [14]. Chen et al. have shown that platelets has an efficient role in the proliferation of stem cells due to the presence of growth factors, but it does not play a role in osteogenic differentiation [15]. In a similar in vitro study, Dohan Ehrenfest et al. also showed that platelets have a significant stimulatory effect on both proliferation and differentiation of stem cells [16].

Our findings have shown that the stem cell and fibroblast conditioned medium can be an easy and promising method for the repair and reconstruction of articular cartilage defects. In the histological evaluation with Masson's trichrome staining, we showed treatment with fibroblast conditioned medium led to an increase in collagen deposition in the repair tissue. Hur et al. stated that stem cells that are exposed to fibroblast culture medium lead to an increase in collagen type 1 secretion. They also showed that the fibroblast conditioned medium plays a role in the differentiation of stem cells into fibroblasts and skin wound healing [17]. Zhang et al. showed that stem cell therapy at the defect site result in significant glycosaminoglycan and type II collagen formation in the new cartilage [18]. The data of this study showed that the stem cell conditioned medium could lead to the deposition of collagen and glycosaminoglycan more effectively compared to stem cell therapy alone.

Endo et al. have shown the role of FGF-2 in chondrogenic differentiation of stem cells and articular cartilage regeneration [12]. Cucchiarini et al. showed in vitro and in vivo the role of FGF-2 in stimulating the proliferation of chondrocytes, filling the defect site, structural and morphological improvement of osteochondral defects in the rabbit knee joint [19]. In this study, it has been seen that the conditioned medium of stem cells and fibroblasts has a more effective role in the proliferation of cells and the formation of cartilage-like cells. Which is probably related to the growth factors in the conditioned medium. Also, the maintaining matrix homeostasis and increasing autophagy, and delaying the progression of osteoarthritis [20], anti-apoptotic [21] properties of the conditioned medium were also seen in the studies.

One of the limitations of this study was the identification of the type of collagen by immunohistochemical evaluation and evaluation of the repaired tissue in a longer period of time.

4. CONCLUSION

Based on the histological findings, the conditioned medium with stem cells had better results compared to the conditioned medium with fibroblast and PRP in creating hyaline-like reparative tissue, collagen deposition and glycosaminoglycan synthesis. The effectiveness of the role of conditioned medium compared to cell therapy may be due to the growth factors present in them.

5. MATERIALS AND METHODS

5.1. Isolation, expansion and characterization of BM-MSCs

In this experimental study, mesenchymal stem cells were obtained from bone marrow and fibroblasts from the skin of an adult male Wistar rat that was anesthetized with ketamine (50 kg/kg) and xylazine (5 mg/kg). To isolate and collect mesenchymal stem cells from the bone marrow, the femoral bone marrow were transferred into the Falcon tube using the flushing technique. Then the cell suspension was washed twice with phosphate-buffered saline (PBS) containing antibiotics penicillin, streptomycin, and amphotericin B. After washing and disinfecting, the cell suspension was centrifuged and then transferred into the flask.

To isolate fibroblasts, the skin of the groin area of the rat was first disinfected and its hair shaved. Then a part of the skin is completely removed. After separating the dermis from the epidermis, dermis is washed three times with phosphate-buffered saline (PBS) containing antibiotics penicillin, streptomycin, and

amphotericin B. Then the dermis is divided into small pieces and planted on the bottom of the plate by explanation method.

Then, for both cells, they were cultured inside the plate or flask by adding Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) supplemented with 2 mM L-Glutamine, 10% foetal bovine serum, 1% penicillin, streptomycin, and amphotericin B at 37°C and in a humidity 55% and 5% CO_2. The culture medium was changed every 3 days. When the colonies of fibroblasts and mesenchymal stem cells were well expanded, the cells were trypsinized and transferred to a new dish for further expansion. Cells were cultured until passage 4. Flow cytometry was used to identify mesenchymal stem cells and determine surface markers (CD34, CD44, CD45 and CD105).

5.2. Collection of condition medium

When the stem cells and fibroblasts of passage 4 in the flasks reached the confluence of 75, the medium was removed and the medium without serum was added to the flask. Then 24 hours later, the conditioned media was collected. After centrifugation at 4°C and 3000 rpm for 10 minutes, the conditioned medium was frozen to be used for intra-articular injection when necessary [22, 23].

5.3. Preparation of PRP

PRP was prepared based on the previous research [24]. The blood sample was collected from the tail vein of the rat and transferred into sterile test tubes, then immediately centrifuged (3000 rpm, 10 min). The clot in the middle layer of the test tube was removed and the red blood cells at the bottom and acellular plasma at the top of the tube were discarded. The PRP clots were frozen for later use.

5.4. Animals

In this experimental study, thirty-five adult male Wistar rats with a weight of 200-250 g are obtained from the Animal Research Center of Mazandaran University of Medical Sciences, Sari, Iran. In order to adapt to the environment, these animals were kept in their cages for 1 week with free access to food and water and under standard environmental conditions (12 hours of light and 12 hours of darkness, temperature 26-28^{0}C, and humidity 50%). This experimental study was approved by the Institutional Animal Ethics Committee of the Mazandaran University Medical Sciences (ID: IR.MAZUMS.4.REC.1401.14094).

5.5. Induction of knee joint cartilage defect in a rat model

12 hours before anesthesia, the animals' food was stopped. To induce articular cartilage defects, rats were first anesthetized by intraperitoneal injection of ketamine and xylazine. Rat knee joints under aseptic conditions were opened using the medial parapatellar method. Osteochondral defects were created with 2 mm in diameter and 2 mm in depth on the intertubercular groove of the femur [25]. The defects were entirely washed with a normal saline solution. After inducing an articular cartilage defect, the incision on the inner side of the patella was closed with sutures, and gentamicin was injected intramuscularly to prevent infection. No bandages or splints were used to immobilize the limbs. After the end of anesthesia, the animals walked freely without any restrictions. During the postoperative period, the general condition of the animals and any signs of knee infection were evaluated macroscopically. 4 weeks after surgery, the animals were anesthetized with overdose of ketamine and xylazine and sacrificed. The distal femurs were harvested, and the samples were fixed. If an infection developed in the knee, they were excluded from the study.

5.6. Study design

Animals are randomly divided into 8 groups (5 rats in each group).

1) **Control** group: no articular cartilage defects are created in the knees of the animals.
2) **Sham** group: Only the joint capsule was opened and sutured again.
3) **Untreated** group: only articular cartilage defects are created in the knees of animals.
4) **DMEM** group: articular cartilage defect is created in the knee of animals and DMEM is injected into the joint.
5) **Stem cell** group: After inducing the defect model in the knee joint, they received 300,000 stem cells.
6) **Stem cell conditioned medium (SCCM)** group: After inducing the defect model in the knee joint, they received conditioned media of mesenchymal of stem cells.
7) **Fibroblast conditioned medium (FCM)** group: After inducing the defect model in the knee joint, they received conditioned media of fibroblast cells.
8) **Platelet rich plasma** (**PRP)** group: After inducing the defect model in the knee joint, they received PRP.

The Conditioned medium and PRP were prescribed in the amount of 10 microliters.

5.7. Histological evaluation

The samples of repaired articular cartilage tissue were fixed in 10% formalin, then decalcified, dehydrated in alcohol series, clarified in xylene, and embedded in paraffin for routine histological sectioning. The sections of 5µm thickness were stained with hematoxylin and eosin (H&E), Masson's trichrome, and PAS (Periodic Acid Schiff) staining according to standard methods. Then the histopathological slides were evaluated under light microscopy (Olympus, Japan) with magnification of 40×. Sections were blindly analyzed by histologist. The tissue structure, glycosaminoglycan, and collagen of the slides were investigated. Specimens were graded conforming to a histological scale described by O'Driscoll with slight differences. Particularly, Masson's trichrome staining was used instead of toluidine blue to determine regenerated cartilage tissue from normal cartilage tissue. This scale consists of eight categories and scores from 0 to 22 [26,27].

5.8. Statistical analysis

Results were expressed as mean ± SD. All statistical comparisons were performed by nonparametric analysis Kruskal-Wallis test. *P*-value less than 0.05 ($P < 0.05$) was considered statistically significant (Prism software, USA).

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