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Research Article

HISTOLOGICAL EVALUATION OF SYNOVIAL FLUID EFFECT ON BONE DEFECTS IN RABBIT STIFLE JOINT

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ABSTRACT
The aim of this study was to investigate the effect of synovial fluid on intra-articular bone defects. In this experiment, twelve mature New Zealand rabbits were used. A defect with the size of 4 mm was created in the condyle (experimental group, n = 6) and epicondyle (control group, n = 6) regions of the left and right stifle joint of the rabbits with a hand drill that continued to bone marrow, respectively. Histological examinations were performed on the 30th and 60th days after the operation. Histological evaluations were made according to the scoring system reported by Heiple *et al.*, (1987). At 30 days, the mean histological scores were 6.0 ± 0.894 and 3.00 ± 1.095 in the control and experimental groups, respectively ($P < 0.05$). On the 60th day, the scores were 10 ± 0.632 and 6.17 ± 1.722 in the control and experimental groups, respectively ($P < 0.05$). Histological examination revealed significantly higher scores in the control group than in the experimental group at 30 and60 days. The results indicate that synovial fluid has an inhibitory effect on intra-articular bone defects compared with extra-articular defects.

Keywords: *Histological, Synovial Fluid, Bone, Rabbit*

INTRODUCTION
Bone is a dynamic biological tissue that includes cells that are metabolically active and in a rigid framework (Kalfas, 2001). Unlike other tissues in which the healing process includes the formation of a connective tissue scar with low quality, bone tissue is regenerated and acquires pre-fracture properties (Giannoudis *et al*., 2007).
Bone healing is a complex process of bone regeneration, which includes a series of well-coordinated biological and cellular events.
During bone healing, a series of signalling molecules initiate the bone formation chain through the intramembranous and endochondral pathways (Dimitriou *et al*., 2006). This process recruits mesenchymal stem cells (MSCs) and bone precursors through the blood vessels and periosteum tissue and induces them to proliferate and differentiate into osteoblasts (Pountos and Giannoudis, 2005).
These signalling factors include pro-inflammatory molecules such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), growth factors such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), and matrix metalloproteases (MMPs) molecules (Marsell and Einhorn, 2011).
These molecules, along with inhibiting factors and a variety of cells located in the affected area and blood circulation, play a central role in improving the various stages of healing (Dimitriou *et al*., 2006).
Thus, the balance between messaging molecules in bone formation and their inhibitors is an important determinant in bone regeneration and development.
Synovial fluid is a clear liquid that is produced by fibroblasts in the synovium. Joints are considered as a major and unique challenge in the healing process due to the existence of destructive enzymes in synovial fluid (Ge *et al*., 2006).
With the onset of inflammatory processes, cytokines such as IL-1 and TNF-α, enzymes such as MMPs that have had catabolic side effects are produced in large quantities in the synovial fluid (Goldring, 2001). Many of these molecules impact on the soft and hard tissues within the joint space.

Therefore, the identification catabolic and anabolic molecules in the process of bone repair are very important.

In this study, we histologically evaluated the short and long term effects of synovial fluid on bone repair in intra-articular (condyle) and extra-articular lesions (epicondyle), which were far from the synovial fluid effects.

MATERIALS AND METHODS

Twelve skeletally mature male New Zealand White rabbits (6 months skeletal maturity; 3–3.5 kg) were used for this study. The animals were housed in individual cages in a temperature-controlled room (20°C–25°C).

The study protocol was under the supervision of the Animal Care Committee of the Iran Veterinary Organization. The study was approved by the University Ethics Committee.

General anaesthesia was induced by an intramuscular injection of ketamine hydrochloride (35 gk/mg, Alfasan, Woerden-Holland) and xylazine hydrochloride (3 mg/kg, Alfasan, Woerden-Holland).

All operations were performed on the right and left stifle joints of the rabbits. On the left side, using aseptic procedures, the joint was entered after a standard medial parapatellar approach and incision on the joint capsule, and then the patella was dislocated laterally. A chondral defect, 4 mm in diameter, was created on the medial femoral condyle (experimental group) using a dermal biopsy punch. Defects were drilled with a hand drill (4-mm drill bit).

The joint capsule was closed with Vicryl 4/0 (polyglycolate coated, Supa Medical Devices, Iran). On the right side, a medial parapatellar approach (no entry in the joint capsule) was made. An extra-articular bone defect, 4 mm in diameter, was created on the medial epicondyle (control group) with a hand drill. In both groups, bone drilling extended to the bone marrow space.

The surgical site was irrigated with saline solution to avoid thermal necrosis and remove bone fragment. After operation, rabbits were housed in individual cages to move freely and were nourished with standard feed for one week.

Preoperatively, all rabbits received an intramuscular injection of antibiotic (oxytetracycline hydrochloride 5%, 15 mg/kg; Razak Laboratory, Tehran, Iran) and another two days after the operation. Pain relief was provided by administrating ketoprofen (10 mg/kg, Razak Laboratory, Tehran, Iran) intramusculary for two days after the operation.

Histology and Staining

All animals were euthanized with a high-dose intra-peritoneal sodium pentobarbital injection. Histologically, the rabbits were investigated at 30 days (n = 6) and at 60 days (n = 6) post operation, respectively.

Distal portions of each femur were detached and fixed for one week in neutral-buffered 10% formalin solution and then decalcified in 10% ethylene-diamine-tetra-acetic acid (EDTA) solution for four weeks, dehydrated and embedded in paraffin. A section of 4 micrometre thickness was taken (perpendicular to the long axis of bone).

Sections were stained with hematoxylin-eosin and Masson's trichrome. Specimens were examined under a light microscope.

Semiquantitative Histological Scoring

Union (0-4 score), spongiosa (0-4 score) and bone marrow (0-4 score) were graded according to the histological scoring system proposed by Heiple *et al.*, (1987) (Table 1).

Statistical Analysis

Statistical analyses were performed using a commercial software package, SPSS version 15. The mean and standard deviation were calculated for both the individual categories and the total score of each of the groups.

Histology scoring was analysed using a non-parametric Mann–Whitney U test. P values of < 0.05 were regarded as statistically significant.

Table 1: Histological Scoring System

Category	Point
Union (the Highest Score is 4)	
No Sign of Union	0
Fibrous Union	1
Osteochondral Union	2
Bone Union	3
Complete Reorganization	4
Spongiosa (the Highest Score is 4)	
No Sign of Cellular Activity	0
Early Bone Formation	1
Active New Bone Formation	2
Reorganized Spongiosa Formation	3
Complete Reorganized Spongiosa	4
Bone Marrow (the Highest Score is 4)	
Not Available	0
Detection of Fibrinous Material	1
Defect Occupying more than Half	2
Fully Occupying the Red Bone Marrow	3
Adult Type Fatty Marrow	4
Sum of Histologic Scores	12

RESULTS AND DISCUSSION

Histological Observation

30 Days after the Operation

The defect was filled with plenty of fibrous tissues and fibrocartilaginous callus in the condyle site (experimental group). No osteogenic activity was observed in the affected area (Figure 1), while the defect site in the epicondyle region had a better repair process than the condyle region. Histological findings revealed a particular difference in callus formation in the two groups ($P = 0.002$; Table 2). In the defect site of the epicondyle group, the absorption and conversion of cartilage to woven bone was observed without reconstruction of bone marrow. In addition, new trabecular bone was found and covered with osteoblasts cells (Figure 2). Histological findings showed that bone repair was better and faster in the control group than the experimental group 30 days after injury ($P < 0.05$; Table 2).

60 Days after the Operation

Histological findings in the condyle area showed that callus fibrosis had converted into a cartilaginous callus and the beginning of the ossification processes is evidenced by the formation of new woven bones. The space between the trabeculaes of the bone was filled by a newly formed bone marrow (Figure 3). The findings in the epicondyle region demonstrated that cartilaginous callus is small and fading. Improved bone quality and repair processes were observed in the control group than in the experimental group ($P = 0.02$; Table 3).

Table 2: Results of the Histological Evaluation on the 30th Postoperative Days (Mean ± SD)

Variable	Control (Epicondyle) (n=6)	Experimental (Condyle) (n=6)	P Value
Union	1.67 ± 0.516	1 ± 0	0.019
Spongiosa	2.5 ± 0.548	1.33 ± 0.816	0.026
Bone Marrow	1.83 ± 0.753	0.67 ± 0.516	0.016
Overall	6.0 ± 0.894	3.00 ± 1.095	0.002

SD Standard Deviation

The existence of pink, calcified cartilage areas and newly formed bone matrix was evident. Lamellae bone is newly renovated and can be seen with woven bone. The newly formed cancellous bone with the organized bone marrow is clear (Figure 4).

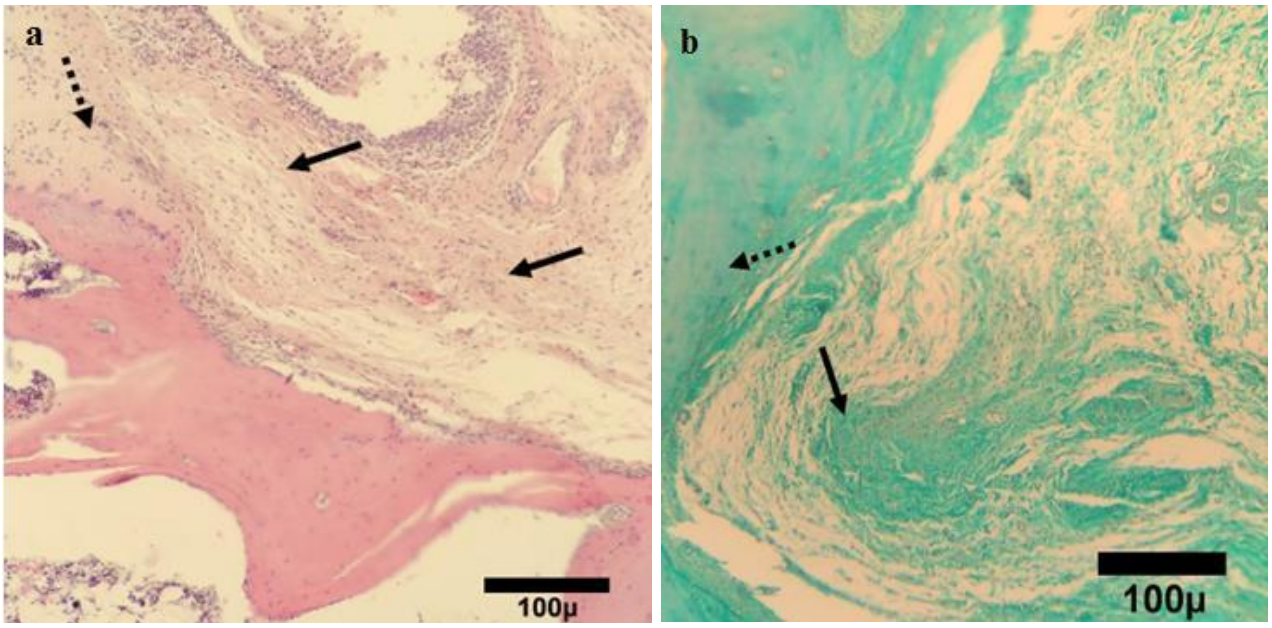

Figure 1: Histologic Analysis of Bone Defect Healing on the 30th Postoperative Days in the Experimental (Condyl) Group a: Staining Hematoxylin–Eosin, Magnification ×100; b: Staining Masson's Trichrome, Magnification ×100
Arrows, Fibrous Callus; Dotted Arrows, Articular Cartilage

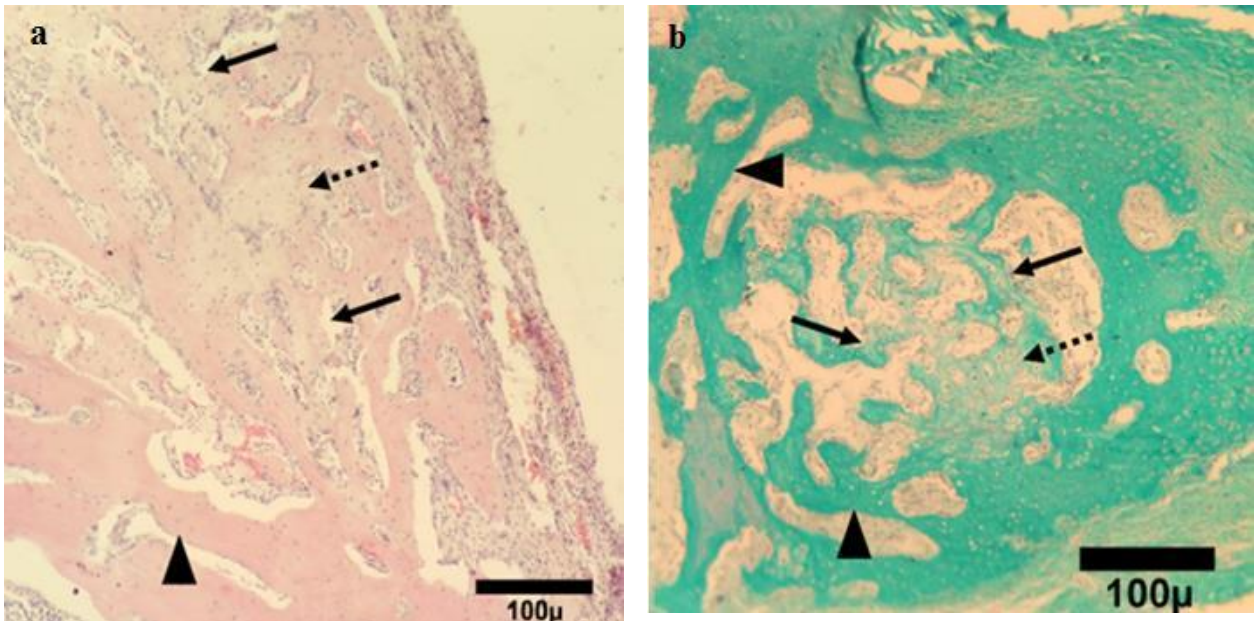

Figure 2: Histologic Analysis of Bone Defect Healing on the 30th Postoperative Days in the Control (Epicondyl) group a: Staining Hematoxylin–Eosin, Magnification ×100; b: Staining Masson's Trichrome, Magnification ×100
Arrows, Woven Bone; Dotted Arrows, Cartilage Callus; Arrowheads, Lamellar Bone

Histological Grading

At 30 days, the mean histological scores were 6.0 ± 0.894 and 3.00 ± 1.095 in the control and experimental group, respectively (Table 2). On the 30th day, statistical testing showed that the mean scores in the control group were significantly superior to those in the experimental group ($P < 0.05$).

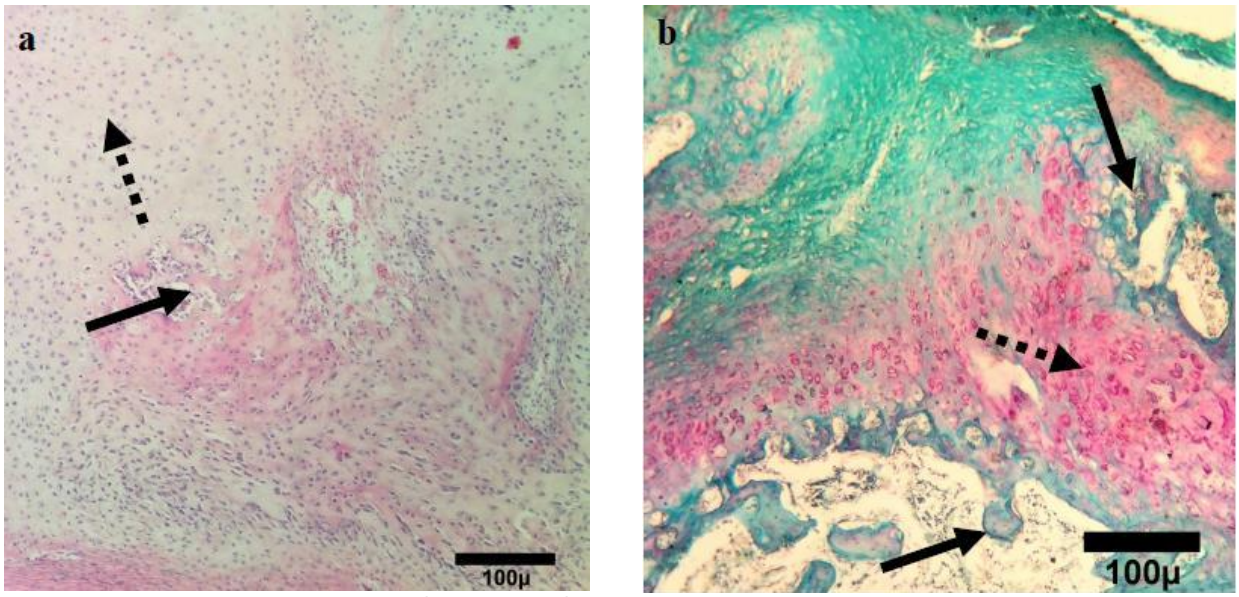

Figure 3: Histologic Analysis of Bone Defect Healing on the 60th Postoperative Days in the Experimental (Condyl) Group a: Staining is Hematoxylin–Eosin, Magnification ×100; b: Staining Masson's Trichrome, Magnification ×100; Arrows, Woven Bone along with Lines of Osteoblasts; Dotted Arrows, Cartilage Callus

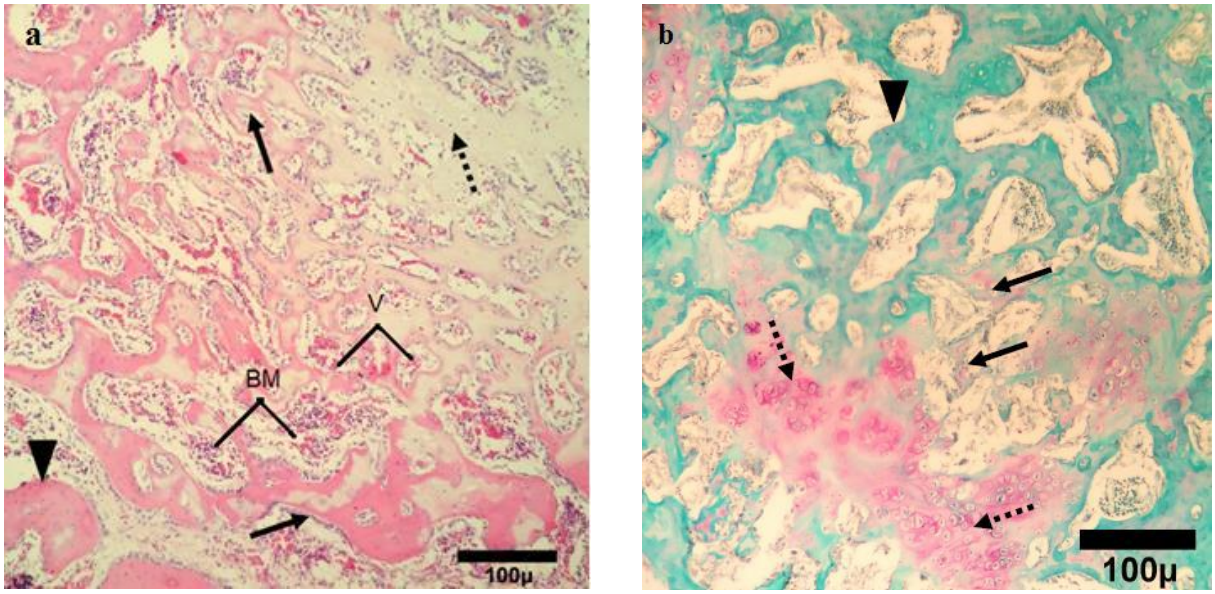

Figure 4: Histologic Analysis of Bone Defect Healing on the 60th Postoperative Days in the Control Group a: Staining Hematoxylin–Eosin, Magnification ×40; b: Staining Masson's Trichrome, Magnification ×100;
Arrows, Woven Bone; Dotted Arrows, Cartilage Callus; Arrowhead, Lamellar Bone; V, Vessels; BM, Bone Marrow

At 60 days, the mean histological scores were 10 ± 0.632 and 6.17 ± 1.722 in the control and experimental group, respectively (Table 3). On the 60th day, the values in the control group displayed statistically significantly (except bone marrow score, P = 0.203) higher histological scores (P < 0.05).
On the 30th and 60th postoperative days, the mean histological evaluation scores were 5.67 ± 0.816 and 9.33 ± 0.816 respectively, in the control group (Table 4). Statistical analysis using the Mann-Whitney test showed a significant difference between 30 and 60 days in the control group (P < 0.05).
On the 30th and 60th postoperative days, the mean histological evaluation scores were 3.00 ± 1.095 and 6.17 ± 1.722, respectively, in the experimental group (Table 5). Statistical testing showed that the histological scores (spongiosa, bone marrow and overall) were different in both cases (P < 0.05), whereas, union value was similar at the 30th and 60th day (p > 0.05).

Table 3: Results of the Histological Evaluation on the 60th Postoperative Days (Mean ± SD)

Variable	Control (Epicondyle) (n=6)	Experimental (Condyle) (n=6)	P Value
Union	3.33 ± 0.516	1.17 ± 0.408	0.002
Spongiosa	3.17 ± 0.753	2.33 ± 0.816	0.032
Bone Marrow	3.50 ± 0.548	2.67 ± 1.211	0.203
Overall	10 ± 0.632	6.17.± 1.722	0.002

Table 4: Results of the Histological Evaluation in Control Group on the 30th and 60th Postoperative Days (Mean ± SD)

Variable	on the 30th Day (n=6)	on the 60th Day (n=6)	P Value
Union	1.67 ± 0.516	3.33 ± 0.516	0.003
Spongiosa	2.5 ± 0.548	3.17 ± 0.753	0.016
Bone Marrow	1.83 ± 0.753	3.50 ± 0.548	0.006
Overall	5.67 ± 0.816	9.33 ± 0.816	0.003

Table 5: Results of the Histological Evaluation in Experimental Group on the 30th and 60th Postoperative Days (Mean ± SD)

Variable	on the 30th Day (n=6)	on the 60th Day (n=6)	P Value
Union	1 ± 0	1.17 ± 0.408	0.317
Spongiosa	1.33 ± 0.816	2.33 ± 0.816	0.044
Bone Marrow	0.67 ± 0.516	2.67 ± 1.211	0.008
Overall	3.00 ± 1.095	6.17 ± 1.722	0.004

Bone healing in the epiphyseal region is different to that in the diaphyseal region. In the diaphyseal region it occurs endosteally and periosteally, while it only occurs endosteally in the epiphyseal and metaphyseal areas (Uhthoff and Rahn, 1981). They have shown that bone destruction in the rabbit metaphyseal region is endosteally healed without external callus formation. In our experimental model, both defects were in the epiphyseal region, and thus, the process of bone repair in both areas is the same. Histologically, we observed cartilaginous callus formation and its conversion to bone, which shows that osteogenesis process was performed at the site of both defects through endochondral ossification. Successful repair of the bone tissue dependent on osteogenesis. The process of osteogenesis is related to the presence of a sufficient number of osteoblasts to recruit the bone marrow mesenchymal progenitor cells, endosteum and periosteum (Shirley *et al.*, 2005). Progenitor cells in the periosteum tissue have the ability to produce bone and cartilage, while endosteum tissue is limited to osteogenesis (Hankenson *et al.*, 2014). Although

the synovial fluid contains mesenchymal progenitor cells, it has chondrogenic potential in terms of its performance and does not play a role in osteogenesis (De Sousa *et al*., 2014). Murphy *et al*., (2002) reported that bone marrow-derived mesenchymal cells in damaged joints have low proliferative capacity and chondrogenic differentiation potential, but their osteogenic capacity does not change. In our research, in the epicondyle region only the periosteum, endosteum and bone marrow are the origin of bone progenitor cells, while in the condyle area only the endosteum tissue and bone marrow are the origin of bone progenitor cells. It is thus suspected that the healing process in the epicondyle region is faster and better in both the short and long-term than in the condyle region, although the contribution of each of these factors in the process of osteogenesis is not completely clear.

Bone regeneration is a complex process that is regulated by the interference and emergence of growth factors and biochemical enzymes (Probst and Spiegel, 1997). These growth factors encourage and stimulate the migration of mesenchymal progenitor cells to the damaged areas. In lesions to the joint and its structures, the content and expression of growth factors such as BMPs are reduced. These molecules stimulate osteogenesis and improve the bone formation process in the endochondral ossification process (De Luca *et al.*, 2001). The BMP expression in the articular lesions is reduced compared to normal joints (Bramlage *et al*., 2006). Therefore, the process of osteogenesis is also delayed. This finding agrees with our results that show that the process of osteogenesis is slower inside the joint compared outside joint.

The synovial fluid contains inflammatory cytokines. In the physiological state, the anabolic and catabolic balance is established between these factors. Any damage to the joint capsule (surgical incision) or intra-articular structures (damage to the condyle) causes an immediate increase in the concentration of inflammatory cytokines, and an increase in the expression of genes and leads to the changes which occur in osteoarthritis. After a few weeks, the immune responses had subsided, and an increase was observed in the amount of osteogenic factors (Fahlgren *et al*., 2006). Two of the most important inflammatory cytokines in the bone repair process are IL-1 and TNF-α. Various researches have reported that pro-inflammatory cytokines are involved in the production of osteoclast, bone lysis and inhabitation of osteogenesis (Jung *et al.*, 2014).

Cho *et al*., (2006) showed that TNF-α lead to osteogenic induction from MSCs in vitro. Xu *et al*., (2014) research *in vitro* and *in vivo* showed that IL-1 and TNF-α stimulate intracellular adhesion molecule-1 and retard the osteogenic differentiation of mesenchymal progenitor cells in the synovial fluid. The former results are unlike our findings, and the latter results are in agreement with our results. However, it should be noted that we did not measure the amount of IL-1 and TNF-α in this experiment, but the incision on the joint capsule and damage to the articular cartilage probably causes a severe and strong immune response.

According to our histological evidence of condyle models at days 30 and 60, the inhibitory effects of inflammatory cytokines can be explained on osteoblasts. Synovial fluid also contains degrading enzymes, such as MMPs, whose acts are inhibited by tissue inhibitors of metalloproteases (TIMP). In physiological states, the expression of these molecules in the synovial fluid is low, and there is a balance between these molecules. However, in pathological states the expression of these molecules is unregulated. It has been proven that many MMPs, including MMP-2, 9 and 13, participate in the development and regeneration of bone (Ortega *et al*., 2004).

However, in our findings, the osteogenesis process was inhibited in synovial fluid. Colnet *et al*., (2003) reported that MMP-9 gene evacuation in mice results in delayed bone healing due to the continuous production of cartilage in the damaged site. Although there is evidence of the restorative effects of MMPs and its inhibitors on hard and soft tissues, most evidence indicates their destructive and catabolic effects on the bone. MMP has inhibitory effects on the osteogenesis process; MMP-1 over expression reduces BMP levels in synovial fluid, and this indicates that MMP has the opposite effect to BMP (Hayami *et al*., 2011). In general, according to the aforementioned findings, the balance between MMPs and TIMPs is necessary in the healing of bone defects. In our research, we observed the inhibitory process of osteogenesis in intra-articular lesions. Therefore, our test model showed that the presence of MMPs in synovial fluid inhibits the formation and development of new bone and this strengthens our findings.

The role of synovial fluid in the healing process of intra-articular lesions is contradictory. Lunborg and Rank's (1978) studies on the healing tendon in the knee joint of rabbits showed that tenocytes survive in synovial fluid and hold resilience capacity. Biddulph *et al*., (2014) results were agreed with our finding and showed that intra-articular lesions in baboon animal models cause new bone formation. However, though our work shows no bone repair in intra-articular lesions on day 30 after surgery, it does show the healing process of intra-articular bone defects in the long term.

In reconstruction of the front cruciate ligament, Fuch *et al*., (2012) concluded that when synovial fluid enters the bone tunnel it has an inhibitory effect on fibroblasts. They further observed that synovial fluid causes dilation of the tunnel. Their studies showed that synovial fluid inhibits osteoblast differentiation, although it may have stimulatory effects on their proliferation, and that the former is necessary for healing and bone formation.

Berg *et al*., (2001) compared the bone healing process in the intra- and extra-articular tunnel. They showed that the tunnel located outside the joint had led to restoration of 99% of the bone defect at weeks 4 and 12. However, healing of the intra-articular tunnel was delayed and incomplete in this period. Sun *et al*., (2012) showed that synovial fluid has inhibitory effects on intra-articular lesions in the rabbit animal model and their results will strengthen our work.

Although several studies were conducted on the impact of synovial fluid on soft tissue, its effects on hard tissue are not well known. In the process of indirect bone healing, mesenchymal progenitor cells entered into the area and were converted into fibrous and fibrocartilage tissues and eventually bone. Presumably, the presence of large amounts of fibrous tissue in the defect location is due to the stimulatory effect of synovial fluid in the proliferation of fibroblasts. On the other hand, the presence of abundant fibrous tissue in the area leads to inhibition of osteogenesis and delayed bone formation (Malhotra *et al*., 2014). Therefore, mitogen stimulation of synovial fluid on fibroblasts does not guarantee a beneficial and stimulating effect on osteogenesis. The histology test results showed that large amounts of fibrocartilage tissue were found 30 and 60 days after surgery in the condylar region that this tissue, delaying the process of osteogenesis in our experimental model.

Although, we tested the influence of various factors on the healing and non-healing of cancellous bone in synovial joints, the contribution of each of these agents is not well understood. This experimental model is appropriate in rabbits, for genetic and cellular studies to determine the role of specific proteins in bone repair.

Conclusion

Our laboratory model showed that the effect of synovial fluid on intra-articular bone defects healing in comparison to extra-articular bone defects is slow and delayed 30 and 60 days after surgery.

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