The effect of mesenchymal stem cells, demineralized bone graft and platelet-rich plasma on osteogenesis in rat tibia defects

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Abstract

Aim: Deformities of the jaw and face are often caused by infection, inflammation, and cystic and neoplastic pathological conditions. Defects with various aetiologies should be repaired promptly using the most appropriate approach to reconstruct the anatomical form. To treat defects, bone grafts with various combinations have been used. In particular, combinations including cellular products to enhance osteogenic properties have been implemented. In this study, we aimed to investigate the effects of different materials and cells on bone defects by using mesenchymal stem cells (MSCs), which are thought to have a positive effect on healing, demineralized bone graft (DMB) and platelet-rich plasma (PRP).

Methodology: We used 55 female rats weighing between 200-250 g, four of which were used to obtain platelet-rich plasma. The remaining animals were divided into five groups. Group I (n = 6) was the operative control group, Group II (n = 24) was given DMB, Group III (n = 24) was given DMB+PRP, Group IV (n = 24) was given MSC+DBG and Group V (n = 24) was given DMB+PRP+MSC applied to rat tibial defects (10 mm x 3 mm x 2 mm).

Results: Statistically significant differences were observed in bone osteoblastic activity in tibia defects among the groups (p<0.05).

Conclusion: Bone regeneration was significantly improved in groups where MSCs were used in combination with DMB and PRP.

Keywords: bone defect, demineralized bone graft, mesenchymal stem cell, platelet rich plasma, rat

Introduction

Treatment of intra-bone defects is improved by the use of techniques that increase the rate of bone regeneration. Examples of techniques that have increasingly been used to remedy bone defects include new graft materials, biostimulator systems, systemically applied vitamins, hormones and mineral supplements, electrical stimulants, growth factors and stem cells (1,2). Among the graft materials used, autogenous bone grafts are generally considered the first choice (3,4). However, autogenous grafts may cause donor site morbidity, thus requiring a second surgical site (5,6). Therefore, cell-based therapies and
materials that stimulate bone formation have become widely popular for the treatment of bone defects. Allografts are graft materials obtained from different individuals with different genotypes. Laurencin et al. described demineralized bone powder as allograft-based bone materials (7). Several studies have demonstrated the efficacy of demineralized bone matrix in treatment of bone defects (8-12). In addition to being osteoinductive, bone matrix also has osteoconductive properties due to its collagen content (13). In addition, bone morphogenic proteins (BMPs) are responsible for the bone-forming properties of demineralized bone matrix (DBM) which includes many proteins. BMPs increase the chemoattraction of cells (MKH) to the environment and promote the differentiation of host mesenchymal stem cells into bone-forming cells (14-16).

The regenerative potential of platelets was demonstrated in the 70s after observing that they support production of collagen, cell mitosis, growth of blood vessels, and production of growth factors responsible for the development of other cells that migrate to the injury site (17). The activation of high density platelets with thrombin results in plasma that is rich in mitogenic factors, referred to as “platelet rich plasma (PRP)” (18,19). In 1997, Whitman et al. were the first to introduce the use of PRP in oral surgical procedures and stated that PRP provides great advantages for developing host bone and osteoprogenitor cells in bone graft (20). In a comparative study in 1998, Marx et al. applied PRP onto cancellous bone grafts used for mandibular defect reconstruction and found that PRP had positive effects on bone healing (21).

In living organisms, cells that have the ability to divide for long periods of time, to self-renew, and to differentiate into specialized cells (plasticity) are called stem cells (22). Depending on the source from which they are obtained, stem cells are classified as embryonic (fetal), or adult (postnatal) stem cells (23). Embryonic stem cells originate from the embryo and are capable of forming all types of tissues (24,25). Adult stem cells are undifferentiated cells present within formed tissues, and that can undergo self-renewal and become specialized cells of the organ from which they originate. These cells are also called somatic stem cells and under special conditions they can also transform into cell types belonging to other tissues (26). Sanchez-Ramos et al. reported that bone marrow stromal cells (BMSCs) can be converted to neurons, and Ferrari et al. reported that these cells may be involved in the repair of skeletal muscle tissue (27,28). Recent studies have shown that BMSCs can be transformed into osteoblasts, chondrocytes, adipocytes, myoblasts, hepatocytes, cardiomyocytes and neural cells (29).

Mesenchymal stem cells (MSCs) obtained from adults are more advantageous than other cells in terms of clinical use because they are easy to isolate and have high potential for differentiation. In addition, the negligible immune reaction following allogeneic transfer makes MSCs the ideal cell type to be used for tissue repair and regeneration in cell therapy applications (30). Lechner and Huss reported that they isolated MSCs from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscles, tooth pulp and periodontium (31). Other studies have shown that these cells can be transformed into connective tissue cells, including bone, fat, cartilage and muscle tissues (32,33).

In this study, using combinations of MSCs, demineralized bone graft, and platelet-rich plasma, we investigated the effects of cell-based therapies on osteogenesis in experimental bone cavities, histologically and immunohistochemically.

Materials and Methods

Test animals
This study was carried out at the Dicle University Health Sciences and Application Centre (DUSAM) with the approval of the Presidency of Experimental Animals Ethical Committee of the Rectorate of Dicle University dated 29.06.2010 and numbered 2010/36.

In this study, we used 55 female rats weighing between 200-250 g, of which four rats were used to obtain PRP. The remaining experimental animals were divided into five groups. While Group I (n = 6) was the operative control group, DMB graft was used in Group II (n = 24), DMB graft and PRP were used in Group III (n = 24), and MSCs and DMB graft were used in Group IV (n = 24), and in Group V (n = 24) MSCs, PRP, and DMB graft were applied to the defects.

Rats were housed individually in suitable cages at 22 ± 2°C, and 12 h dark / 12 h light conditions. Nutritional needs of the animals were met regularly by giving them standard laboratory food and water.

Bone graft material
Grafton® (Osteotech) is demineralized human bone graft. We used it in our experimental study due to its osteoconductive and osteoinductive properties. Although different forms of bone graft are commercially available, the putty form is preferred for ease of application to the defect site.

Preparation of platelet-rich plasma
PRP was obtained by using a platelet-rich plasma kit and a centrifuge device (Heraeus, RA Medical Devices Industry and Trade Inc.). To prepare PRP, four rats were anesthetized by intramuscularly administering a combination of Xylazine HCl (Alfazyne® 5 mg/kg, and ketamine HCl (Alfamine® 35 mg/kg. Once anesthesia was achieved, all the blood of the animal was withdrawn intracardially using a syringe and transferred into sodium citrate tubes. The blood was then centrifuged at 2400 rpm for 10 min in the Laboratory of Biochemistry, Department of Dicle University Medical Faculty. With this method, PRP and PPP (Platelet-Poor Plasma) were separated from the red blood cell fractions. PRP and PPP were transferred to another tube and centrifuged again at 3600 rpm for 15 min to separate them, and a higher concentration of PRP was obtained.
Isolation and culturing of rBM-MSCs

Wistar female rats (8-10 weeks old) were obtained from the Experimental Animal Centre of Kocaeli University (Kocaeli, Turkey). Animal housing and experiments were approved by the local Animal Care Committee according to the institutional guidelines and national animal welfare. The animals were housed under standard conditions for one week prior to use. To establish the MSC culture, the animals were anesthetized with Ketalar (Pfizer, Istanbul, Turkey) and sacrificed by cervical dislocation. Under sterile conditions, femurs and tibiae from each rat were excised, and muscle and connective tissues were detached.

The size of the needle could have an effect on cell viability and functional changes could be induced by the stress of expulsion of the suspension from a narrow bored-sized needle. Thus, the Experimental Animal Centre of Kocaeli University (Kocaeli, Turkey) was designed study 21-G needle size. The ends of the bones were removed, a 21-G needle was inserted into the shaft, and the bone marrow was extruded by flushing with cell culture medium composed of Dulbecco’s modified eagle medium (DMEM) -Earle medium (Life Technologies/GIBCO, Paisley, UK) supplemented with 15% fetal bovine serum (FBS; Life Technologies), 100 IU/ml penicillin and 100 mg/ml streptomycin. The bone marrow was diluted 1:3 with phosphate buffered saline (PBS) and layered over a Histopaque-1077 (1.077 g/ml, Sigma-Aldrich, St. Louis, MO, USA) for gradient centrifugation. The low-density mononuclear cells were collected, washed twice with PBS, counted, and plated in tissue culture flasks at a density of 1.4 × 105 cells/cm2 in cell culture medium. The cells were incubated at 37°C in a humid atmosphere containing 5% CO2 for 3 days. On the third day, red blood cells and other non-adherent cells were removed and fresh medium was added to allow further growth. The adherent cells were grown to 70% confluency and passaged at this stage so as not to impede their proliferative capacity. Further passaging of the cells was performed by seeding 3.0 × 103 cells/ cm².

Surgical technique

A total of 55 rats were used in our study. Four rats were used to obtain PRP, and the remaining subjects were divided into five groups. Anesthesia of the experimental animals was provided by intramuscular injection of 5 mg/kg xylazine hydrochloride and 50 mg/kg ketamine-HCl. Both tibiae of the subjects in each study group were used (Table 1).

Table 1. Study Groups

<table>
<thead>
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<th>Groups</th>
<th>Number of rats</th>
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<tr>
<td>1. Gr1 1. Control</td>
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<tr>
<td>2. Graft only (DBM)</td>
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<td>3. Graft (DBM + PRP)</td>
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<tr>
<td>4. Graft (DBM + MSCs)</td>
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<td>5. Graft (DMB + PRP+ MSCs)</td>
<td>12 (n:24)</td>
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</tbody>
</table>

On the inner side of the right and left hind legs of the rats, soft tissue and periosteum were elevated by making a midline incision parallel to the tibia. A bone defect of 10 mm in length, 3 mm in depth, and 2 mm in width was created in the tibia using a physiodispenser. Different graft materials for each group were implanted in the bone defects that were made. Subcutaneous tissues were then closed with 6/0 vicryl suture, and skin was closed with 5/0 silk suture. To prevent infection, a single dose of antibiotic (gentamicin, 0.05 ml/kg) was injected into the gluteal muscle of each rat immediately after the operation.

Termination of the experiment

In the 2nd, 8th, and 12th week of experiment, one-third of the rats in each group (one rat from Group 1, and four rats from Group II, III, IV and V) were sedated with ketamine-HCl (50 mg/kg) IM anesthesia. The animals were euthanized by injection of 2 cc intracardiac Lidocaine HCl (Jetocaine simplex®).

Histological and immunohistochemical assessment

Following euthanasia, the tibia was dissected out and the soft tissues around it were removed, while leaving healthy bone tissue around the defective area. The specimen was placed in 10% formalin solution. Tissues were stained with Hemotoxylin-Eosin for histological examination at Dicle University, Faculty of Medicine, Pathology Department Laboratory. For immunohistochemical examination, tissues were stained with osteonectin-osteopontin stain by the peroxidase-antiperoxidase method and evaluated using light microscopy.
Statistical analysis

Results were analysed based on positive-negative response of the sections to osteonectin and osteopontin staining, and osteocyte density (Fig. 1 and 2). Statistical analyses were performed using the SPSS 11.5 package program. In addition to using descriptive statistical methods (frequency distribution), chi-square test was used for comparison of qualitative data. The results were evaluated at the p < 0.05 level of significance. Different results were obtained according to the positivity of osteopontin and osteonectin staining. When all groups were compared with each other, ‘p’ values were calculated, and the differences were revealed. Osteopontin and osteonectin enzyme secretions were very low in the 2nd week, but increased in the 8th week, and significantly increased in the 12th week. We hypothesize that the significant differences we observed between the samples taken at different times (Table 2-3) are likely to be due to increased osteogenesis during the recovery period.

When evaluation was made based on osteocyte density, we found that, in the MSCs-treated groups, osteocyte density increased in the early stages and initiated osteoblastic activity. We interpreted this finding as due to rapid and improved osteogenesis in subsequent recovery periods (Fig. 3).

![Figure 1. Osteopontin enzyme expression](image1)

![Figure 2. Osteonectin enzyme expression](image2)
### Table 2: Percentage of samples stained with osteonectin

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Graft</th>
<th>Graft+PRP</th>
<th>Graft+MSC</th>
<th>Graft+PRP+MSC</th>
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<th>Chi-Square</th>
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### Table 3: Percentage of samples stained with osteopontin

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<th>Graft+MSC</th>
<th>Graft+PRP+MSC</th>
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<th>Value</th>
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<td>%</td>
<td>N</td>
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<td>0%</td>
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<td>12.5%</td>
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<td>37.5%</td>
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<td>62.5%</td>
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<td>12 weeks</td>
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<td>37.5%</td>
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<td>45.8%</td>
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*p ≤ 0.05*
Results

When the patients were examined according to the appliance groups, the trabecular structure of the mandibular condyle bone in the Maxillary expansion system group (Group A) was determined as 1.3564±0.0767 before treatment and 1.3352±0.0556 after treatment. The difference in mandibular condyle bone trabecular structure between individuals in Group A before and after treatment was found to be statistically different (p<0.05).

The trabecular structure of the mandibular condyle bone was determined as 1.3354±0.1368 before treatment and 1.1294±0.1652 after treatment in the Twinblock-Monoblock group (Group B). The difference in mandibular condyle bone trabecular structure between individuals in Group B before and after treatment was found to be statistically different (p<0.05).

The trabecular structure of the mandibular condyle bone was determined as 1.2616±0.0949 before treatment and 1.3211±0.0558 after treatment in the Face mask group (Group C). The difference in mandibular condyle bone trabecular structure between individuals in Group C before and after treatment was found to be statistically different (p<0.05).

In individuals in the Chincup group (Group D), the trabecular structure of the mandibular condyle bone was determined as 1.3345±0.1356 before treatment and 1.3443±0.1652 after treatment. The difference in mandibular condyle bone trabecular structure between individuals in Group D before and after treatment was found to be statistically different (p<0.05).

Discussion

Bone grafting is commonly used for the treatment of congenital or acquired bone defects, including those associated with dento-facial deformities. Therefore, researchers are constantly working to improve bone grafting techniques to achieve faster and more efficient bone regeneration.

The ideal bone graft material should have both osteoconductive and osteoinductive properties (34,35). Autogenous bone grafting remains as the gold standard because it demonstrates these features. However, autogenous bone grafting has several disadvantages, including the requirement of a second surgical site, difficulty in forming the correct shape, infection, resorption, and donor site morbidity (34-36). Many materials have been developed and designed to circumvent these disadvantages and to provide an ideal source of bone grafts. The demineralized bone matrix used in our study was specifically developed for the reconstruction of defects. In 1991, Tiedeman and colleagues used DBM in non-fused tibia fractures that they experimentally created in dogs. Histological examination performed 12 weeks post-operatively revealed an abundance of cartilage, endochondral ossification, and new bone formation. However, in the control group, in which the fractures were left empty, fibrous tissue was observed with minimal new bone formation (37). Yamamoto et al. filled the critical size defects in the premaxils of male Wistar rats with 7 mg of DBM, and left the defects in the control group empty. At the end of the study, they reported that, in the non-grafted group, the fractures healed with fibrous tissue and a small amount of bone formation at the periphery, whereas in the group grafted with DBM,
new bone tissue was formed by osseous bridge in 35 days (38).

Clokie et al. reported that when DBM was used, there was significant ossification and bone marrow formation in the critical size cranial bone defect at the end of the 6th week and that, at the end of the 12th week, bone repair was largely completed and the mature bone and areas of bone marrow had increased significantly in comparison to the 6th week. The rate of new bone tissue formation was reported to be 88% at the end of the 6th week and 95% at the end of the 12th week (39). A study by Colnot et al. showed that when DBM was applied to a bone defect of critical size, the defect was filled with new bone twice as fast as the control group (40).

PRP is used extensively in head, neck, oral, and maxillofacial surgery to improve wound healing and tissue regeneration (41). In a double-blinded, randomized, and controlled clinical study, Hassan et al. demonstrated that particulate bone from the torus mandibula healed 12 out of 24 defects. Studies have shown that when PRP was mixed with bone graft material, marginal bone loss was found to be less in the PRP group compared to the group without PRP (42). Eskan et al. compared the augmentations they performed using either the cancellous allograft alone, or by mixing the cancellous allograft with PRP. They found that, in the group in which PRP was used, there was an increase in horizontal bone width, and in the amount of living bone (43). Agarwal et al. showed that the combined use of PRP and decalcified established frozen bone allograft (DFDBA) in intrabone defects was more effective than DFDBA with serum (44). Piemontese et al. compared DBM in particulate form with graft materials consisting of a mixture of viscous DBM + PRP by applying them to periodontal intra-bone defects; while they did not find any statistical difference in terms of pocket depth reduction and attachment gain, they observed that the amount of bone gain in DBM + PRP graft mixture was higher in standardized radiographic measurements (45). In their study, which was carried out on dogs, Kim et al. applied demineralized bone powder either alone, or in combination with PRP, and examined the osseointegration of dental implants histomorphometrically. They found that when demineralized bone powder was combined with PRP, more bone was formed in the defect area around the implant compared to the untreated control group and the group treated with demineralized bone powder alone (46).

The various drawbacks and limitations associated with the methods currently used to treat defects in maxillofacial surgery, and the insufficient results obtained from these treatments, have uncovered a need for new treatment approaches. Tissue engineering has emerged as an alternative approach in terms of improving the results of traditional treatment options by regenerating living and functioning dental structures (47). In one of many studies addressing the use of stem cells in facial surgery applications, Pieri et al. extirpated bilateral mandibular premolar teeth and reconstructed the sockets with autogenous mandibular bone, fluorohydroxyapatite (FHA), PRP + FHA and MSCs + PRP + FHA. By the end of the third month, they observed that the autogenous bone group and the MSC + PRP + FHA group had more living bone areas compared to other groups in the histomorphometric examination of the materials and that the relationship between the graft particles and the new bone was higher (48). Kojasteh et al. conducted a study on rats; they applied combinations of DBM + PRP, DBM + MSCs, biphasic calcium phosphate (BCP) + PRP and BCP + MSCs to their critical size cranial bone defects. In the evaluation performed at the end of the 6th week, they reported that the best bone healing was in the BCP + MSCs group (49). Yamada et al. compared MSCs and PRP mixture with PRP, autogenous cancellous bone and empty defect area. Two months after the dental implants were placed, histological and histomorphometric evaluations were performed on preparations from sacrificed subjects. Mature bone in the desired form and with new vascularity had formed in the groups treated with MSCs + PRP and cancellous bone. In the MSC-PRP group, it was seen that the alveolar bone was more regenerated than the cancellous bone group and the implant-bone connection was tighter. The authors emphasized that PRP induced bone formation, but not to the same extent as MSCs did (50). Ohyaa et al. compared the effect of MSCs and cancellous bone particles on new bone formation in sinus floor elevation operations. For this purpose, MSCs were mixed with cancellous bone and PRP and implanted in 18 rabbits. Mixtures of cancellous bone particles + PRP, and MSCs + PRP were implanted in one of the maxillary sinuses on both sides of the subjects. At the end of the 8th week, it was found that the MSCs + PRP mixture was superior in terms of osteogenesis and bone volume compared to the cancellous bone + PRP mixture (51).

**Conclusions**

In this study, the effects of MSCs, DBM and PRP on osteogenesis were examined at 2, 8 and 12 weeks; our findings indicate that bone regeneration was significant in groups where MSCs were used and that, by the addition of these living cells to DBM and PRP, mature bone structure has formed. Furthermore, osteoinduction not only improved but also accured at an earlier stage in comparison with the other groups. Therefore, we believe that cell-based therapies will greatly enhance bone repair in maxillofacial surgery and will provide significant improvements in reconstructive surgery.

**Acknowledgments:** This study was presented as a full-text oral presentation at the 1st International Dental Research and Health Sciences Congress held between 20-22 May 2021.

**Ethical Approval:** Ethics committee approval was received for this study from Human Ethics Research Committee of the Rectorate of Dicle University dated 29.06.2010 and numbered 2010/36.
Peer-review: Externally peer-reviewed.

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