

## RESEARCH ARTICLE

## The Effect of Rat Adipose-derived Stem Cells in Bone Tissue Regeneration

Tuncay İLHAN<sup>1,a,\*</sup> Cansel G. ÖZGÜDEN AKKOÇ<sup>1,b</sup> Melike ÇETİN<sup>2,c</sup>  
Ece İNCEBIYIK<sup>1,d</sup> Uygur CANATAN<sup>2,e</sup> Hakan SALCI<sup>2,f</sup> Hatice ERDOST<sup>1,g</sup><sup>1</sup> Bursa Uludağ University, Faculty of Veterinary Medicine, Department of Histology-Embryology, TR-16059 Bursa - TÜRKİYE<sup>2</sup> Bursa Uludağ University, Faculty of Veterinary Medicine, Department of Surgery, TR-16059 Bursa - TÜRKİYEORCID: <sup>a</sup> 0000-0002-7327-9319; <sup>b</sup> 0000-0003-0712-0892; <sup>c</sup> 0000-0001-5424-3321; <sup>d</sup> 0000-0002-2740-4262; <sup>e</sup> 0000-0001-9650-0891<sup>f</sup> 0000-0001-6548-8754; <sup>g</sup> 0000-0003-1547-7293

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**Abstract:** Stem cell approaches has been increasing in the conventional medicine. This study was planned to determinate the efficacy of adipose-derived stem cells (ADSCs) on the bone fracture's regeneration by histologically, morphologically and radiologically. Primarily isolated cells were cultivated and identified for the obtaining of the third passage mesenchymal stem cells in the cell culture laboratory. The right tibias (applied ADSCs) were planned as experimental group and the left tibias were studied as control on the clinically healthy 10 rats. The middle diaphysis of the right and left tibias was transversally cut by a thin saw and then fixed by intramedullary pin. Before surgical closure of the incised area, 10<sup>6</sup> ADSCs were injected on an absorbent material, which covered the cut site of the right tibia, in experimental group. At postoperative 45<sup>th</sup> day, cranio-caudal and lateral radiographs of the tibias pointed out that there was callus formation in the caudal and lateral parts of the right tibia. After sacrifice of the animals, preparations were made for histological examinations. The results indicate that there were clear differences in the mitotic activity; and also, the experimental tibias were found to be rich in blood vessel network. As a conclusion, it was found that ADSCs can have a positive potential effect on osteogenesis in bone tissue.

**Keywords:** ADSCs, Bone Tissue Regeneration, Histologically, Radiologically, RatSıçan Yağ Dokusu Kökenli Kök Hücrelerin Kemik Dokusu  
Rejenerasyonuna Etkisi

**Öz:** Kök hücre teknolojisi, geleneksel tıbbi yaklaşımları geliştirmektedir. Çalışmada, yağ doku kökenli kök hücrelerin (ADSC) kemik kırığının rejenerasyon süreci üzerindeki etkinliği histolojik, morfolojik ve radyolojik olarak değerlendirilmesi amaçlandı. Öncelikli olarak; izole edilmiş hücreler, hücre kültürü laboratuvarında üçüncü pasaj mezenkimal kök hücrelerin elde edilmesi için kültürlendi ve tanımlandı. Klinik olarak sağlıklı 10 rat üzerinde; ADSC uygulanan sağ tibialar deney grubu olarak ve sol tibialar kontrol olarak planlandı. Sağ ve sol tibianın orta diyafizi ince bir testere ile enine kesildi. Sağ kaval kemiği üzerinde, deney grubuna emici bir malzeme üzerine 10<sup>6</sup> ADSC enjekte edildi. Deney ve kontrol gruplarında tibia üzerinde kemik kırığı oluşturulduktan sonra intramedüller tespit yapıldı. Operasyondan 45 gün sonra tibianın kranio-kaudal ve lateral grafleri çekilerek; sağ tibianın kaudal ve lateralinde kallus oluşumu dikkati çekti. Sakrifikasyon sonrası histolojik incelemeler için hazırlıklar yapıldı ve mitotik aktivitede belirgin farklılıklar olduğu saptandı. Aynı zamanda deneysel tibiaların kan damarı ağı açısından zengin olduğu görüldü. Sonuç olarak, ADSC'lerin kemik dokusunda osteogenez için olumlu bir potansiyel etki oluşturabileceği saptandı.

**Anahtar sözcükler:** ADSCs, Kemik Doku Rejenerasyonu, Histoloji, Radyoloji, Sıçan

## INTRODUCTION

Mesenchymal stem cells (MSCs) are attractive therapeutic models for regenerative medicine due to their pluripotent features. These are also undifferentiated cells that have capable of self-renewal with symmetric and asymmetric

divisibility for tissue regeneration. In recent years, applications of MSCs have received increasing attention. Easily extracted from bone marrow and fat, MSCs differentiate into different cell lines according to the needs of certain biomedical applications<sup>[1,2]</sup>. MSCs are suitable cell sources for tissue regeneration. Thus, *in vitro* studies have been

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(\*) Corresponding Author

Tel: +90 224 294 1266 Cellular phone: +90 533 248 9131 Fax: +90 224 294 1202

E-mail: [tilhan@uludag.edu.tr](mailto:tilhan@uludag.edu.tr) (T. İlhan)

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focused on usage of MSCs in bone defect and degenerative bone-cartilage disease known as progenitor stem cells [1,3].

MSCs from different tissues exhibit various *in vitro* characteristics including their proliferation capacity and differentiation potential, which influence their applicability [4]. Lendeckel et al. [5] stated that ADSCs have more proliferative capacity than bone marrow derived mesenchymal stem cells (BMSCs). When compared to other sources, adipose tissue is popular stem cell source because it is easily accessible, abundant, and has less painful collection procedure. Adipose tissue derived mesenchymal stem cells (ADSCs) have a capacity of self-renewable, multipotency and transdifferentiate to several different specific cell phenotypes such as cartilage, bone, fat, tendon and muscle tissue [6,7].

Mohamed-Ahmed et al. [8] shown that equal amounts of bioactive factors are given both of stem cells, ADSCs have inferior capacity and immuno-modulatory effects than BMSCs. ADSCs stimulate macrophage cells however inhibit T cells and dendritic cells, inducing angiogenesis, a decrease in apoptosis and fibrosis leads to an increase in anti-inflammation process [(IL-6, IL-10, IL-13), (TNF- $\alpha$ ), (TGF- $\beta$ ), (TLR 2, TLR4), (VEGF), (b-FGF)] [9]. In tissue engineering, MSCs can be considered as an alternative cell source to ADSCs [10]. BMSCs have also some difficulties, such as low MSC concentrations and difficulty in collecting [11,12]. Moreover, the differentiation of ADSCs into osteoblasts makes them preferable cells for bone tissue engineering [13].

The aim of this study was to evaluate the efficacy of ADSCs on the bone fracture's regeneration process by histologically and radiologically.

## MATERIAL AND METHODS

### Ethical Statement

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (Approval no: 2018-02/03).

### Animals

Totally 13 male Sprague Dawley rats (clinically healthy, about 300 g, 3 months-old) were included in the study. Among them, 10 rats were used for establishment of bone regeneration, and 3 rats were used for harvesting adipose tissue.

### Isolation of ADSCs

Approximately 1-1.5 g adipose tissue per rat was obtained under sterile conditions. The non-enzymatic stem cell method was performed for the isolation of ADSCs as reported previously [14-17]. After adipose tissue was rinsed with sterile D-PBS (Cat No: BSS-1006), adipose tissue

fragments were chopped into 2-3 mm thickness and transferred into rat-MSCs specific growth medium (Cat No: RAXMD-03011-440). Growth medium contained 100 units/mL penicillin-100  $\mu$ g/mL streptomycin (Cat No: TMS-AB2-C), 2 mM L-glutamine (Cat No: G7513), and 10% heat-inactivated Fetal Bovine Serum (Cat No: TMS-013-B). Tissue fragments were incubated in 25 cm<sup>2</sup> tissue culture flask under standard culture conditions. The culture medium was replaced with fresh medium once every three days on passage 1, passage 2 and passage 3.

Third-passaged (P3) cells were subcultured in order to collect the cells for stem cell transplantation. For this purpose, the cultured P3 cells were centrifugated at 1000 rpm, 25°C for 4 min. After the supernatant was discarded, the pellet was filtered with 70-micron filter to use MSCs. The 10<sup>6</sup> ADSCs were prepared by insulin syringes to apply the fractured area of the tibias.

### Adipogenic, Osteogenic, Chondrogenic Differentiation

After isolating and expanding ADSCs, P3 cells (7 $\times$ 10<sup>4</sup> cells per well) were seeded within 24 well plates. The standard medium was replaced with the MSCgo™ Adipogenic Differentiation Basal Medium (Cat No: 05-330-1B, 05-331-1-01 and 05-332-1-15); when cells reached 80% confluency. After two weeks, the cells were stained with Oil Red O [18].

ADSCs (1.5 $\times$ 10<sup>6</sup> P3 cells per well) were seeded within 24 well culture plates, and then MSCgo™ Osteogenic Differentiation Medium (Cat No: 05-440-1B) was added, when cells reached 70% confluency. After 21 days, cells were stained with Alizarin Red [18].

ADSCs (1.5 $\times$ 10<sup>6</sup> P3 cells per well) were seeded within 96 well culture plates. The MSCgo™ chondrogenic differentiation basal medium (Cat No: 05-220-1B and 05-221-1D) was added when cells reached 80 confluency. The cells were incubated within the chondrogenic medium for 21 days and stained with Alcian Blue [18].

### Characterization of ADSCs by Immunohistochemistry

Forty thousand ADSCs were seeded on eight-well plastic culture slide for immunostaining. P3 cells were fixed with 4% paraformaldehyde (Merck, Germany, Cat No: 1.04005.1000) for 1 h at room temperature. The cells were incubated with CD 90 (1:900 dilution, Cat number: ab225), CD 105 (1:150 dilution, Cat No: ab156756), CD 45 (1:200 dilution, Cat No: ab8879) and CD 11b (1:200 dilution, Cat No: ab10558) stem cell surface primary for 18 h at +4°C in humidity chamber. The cells were then incubated with secondary antibody and streptavidin-peroxidase (Cat No: TP-125-HL) for 10 min, respectively. Finally, the bound antibody complexes were stained with chromogen solutions under light microscopic determination and then counter stained with hematoxylin [19].

### Surgical Experimental Protocol

In 10 rats, the right tibias were evaluated as experimental group (application to ADSCs) and the left tibias were planned as control. Before surgery, clinical examinations, mediolateral and craniocaudal radiographies of the bilateral tibias were taken in all rats to evaluate cortical and medullar radio-opacity and to calculate the medullar and cortical space thickness of the tibias.

Premedication and induction were performed using xylazine HCl (5 mg/kg, im.) and ketamine HCl (50 mg/kg, im.), respectively. Isoflurane was inhaled to all rats for general anesthesia and maintenance. The animals were restrained in dorsal recumbence and bilateral tibial regions were prepared aseptically for surgery. The medial longitudinal incisions were made, and medial surfaces of the tibias were exposed following to dissections of the soft tissues. Hemorrhages were controlled with electro-coagulation. The middle diaphysis of the right and left tibias was transversally cut by a thin saw, and then a suitable diameter (0.5-1 mm) Kirschner pin was intramedullary inserted for fixation to the tibias. The surgical incisions of the left tibial regions were closed routinely. Moreover, on the right tibia,  $10^6$  the ADSCs was injected on an absorbent material and this material was covered circumferentially on the cutting surfaces of tibia, were applied in groups (Fig. 1). All soft tissues and skin incisions were sutured routinely.

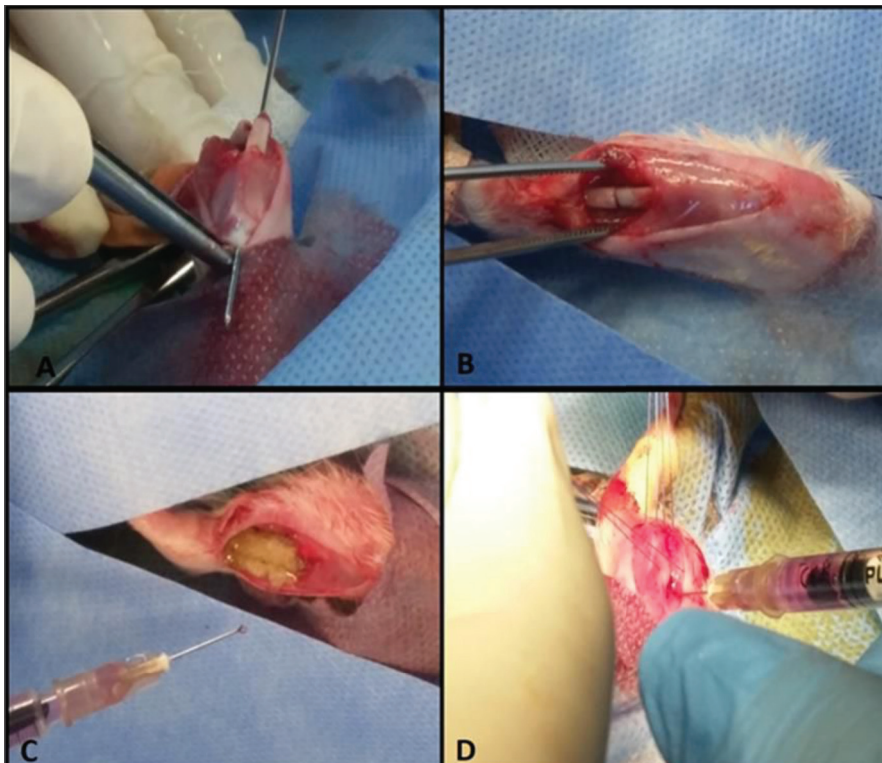
Postoperatively, carprofen (5 mg/kg, sc, qd) and cefazolin Na (20 mg/kg, im, bid) were administered as analgesic and

antibiotic for 5 days, respectively. The skin sutures were taken at postoperative 10<sup>th</sup> day.

Clinical examinations (general condition, inspection and palpation of the surgical area) of the rats were routinely performed at each week in order to evaluate the health status of the rats and to assess the local surgical area regarding tissue healing. At the end of the planned study time (2 months), tibial regions of all rats were clinically examined again, and then all rats were decapitated to obtain the tibias of the rats for histological examinations, as follows.

### Histological Examination

After decapitation, the tibias were quickly harvested and the bone tissue cut into small pieces. Specimens were fixed in 10% neutral buffered formalin for 3 days. After the fixation of specimens, they were decalcified in ethylenediaminetetraacetic acid (EDTA) solution. Until the specimens were soft enough to allow cutting, solution was changed day by day. Depending on the size of the samples, this process took two weeks or more [20,21]. When decalcification was complete, specimens were briefly rinsed in water. After dehydration in 70% ethanol, 80% ethanol, 96% ethanol, and absolute ethanol, specimens were embedded in paraffin wax and then 6-7  $\mu$ m thickness tissue sections were cut and stained with Crossman's triple staining method [22]. Histology sections were examined micro-scopically (Nikon® Eclipse 80i Microscope, Netherlands). Photographs were taken with attached camera (Nikon®, Digital Sight DS-L1).



**Fig 1.** The middle diaphysis of the tibia was transversally cut by a thin saw, and then a 0.5-1 mm Kirschner pin was intramedullary inserted for fixation (A, B). On the right tibia, the applications in groups were injected the ADSCs on an absorbent material which was covered circumferentially on the cutting surfaces of the tibia (C, D)



## RESULTS

### Characterization and Differentiation of the ADSCs

Lipogenic, osteogenic, chondrogenic differentiation capability, positive and negative characterization were used to identify and confirm the ADSCs. On day 3; ADSCs were observed to adhere to culture flasks (Fig. 2-A) and contained both fibroblast-like cells with spindle shape and migrating cells with round shape by inverted microscopy (Fig. 2-B). The first week isolation of ADSCs, the mononuclear cells are adhered to the flask. ADSCs reached 80% confluency on day 7-8. The second and third passage of ADSCs reached 80% confluency on day 13-18 (Fig. 2-C) respectively. P3 cells were determined by lipogenic, osteogenic and chondrogenic differentiation capability *in vitro* that detected using Oil red O, Alizarin Red and Alcian Blue staining, respectively by inverted microscopy (Fig. 3-A,C). ADSCs are characterized by the presence or absence of certain surface markers by the expression of CD 90, CD 105 (Fig. 4-A,B) and CD 11b, CD 45 (Fig. 4-C,D) respectively. ADSCs were showed positive reaction for CD 90, CD 105 and negative reaction for CD 45, CD 11b, as assessed by immuno-histochemistry.

### Histological Results of the Bone Histogenesis

In postoperative 2<sup>nd</sup> months, new bone formation areas were examined histologically in the fractured parts of tibias, and they were determined normal in both tibias with triple stain. The morphology of the cells in the new bone formation areas commonly showed mitotic activity

of the osteoblast cells around the fractured part of the tibias. In addition, the proliferation of the osteoblast cells increased in the callus zone especially experimental tibias than control tibias (Fig. 5), and also bone matrix was strongly eosinophilic in experimental tibias (Fig. 6). The eosinophilic reactions were weaker in the controls than the experimental tibias, two months after operation (Fig. 6). The morphology of the cells pointed out common mitotic activity and there were much more blood vessels in the new bone formation areas in experimental tibias (Fig. 5).

### Clinical and Radiological Results

Clinically, there were no clinical abnormalities such as local tissue damage, ecchymosis and secondary surgical wound complications in the rats of groups at the postoperative early stage.

Radiographs taken on postoperative 21<sup>st</sup> day revealed radiopaque appearance (callus formation) around to fractured parts of tibias. In the postoperative 45<sup>th</sup> days' radiographs, there was callus formation around to fractured line, and the fractured line was clearly determined in these radiographs, as well (Fig. 7). At 45<sup>th</sup> day, craniocaudal and lateral radiographs of the tibias in a rat, it might be clearly seen that there was callus formation in the caudal and lateral parts of the right tibia (arrows) and bilateral visible fractured lines.

## DISCUSSION

The stem cell transplantation system for osteogenesis

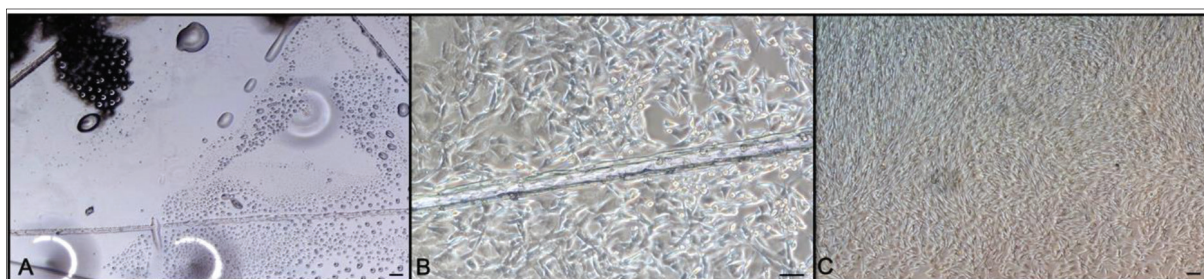


Fig 2. Microscopic evaluation of ADSCs: On day 3, isolation of ADSCs (A); ADSCs were detected with fibroblast-like cells morphology and migrating cells (B); On day13- 18, ADSCs reached 80% confluency (C) (Bar: 100 µm)

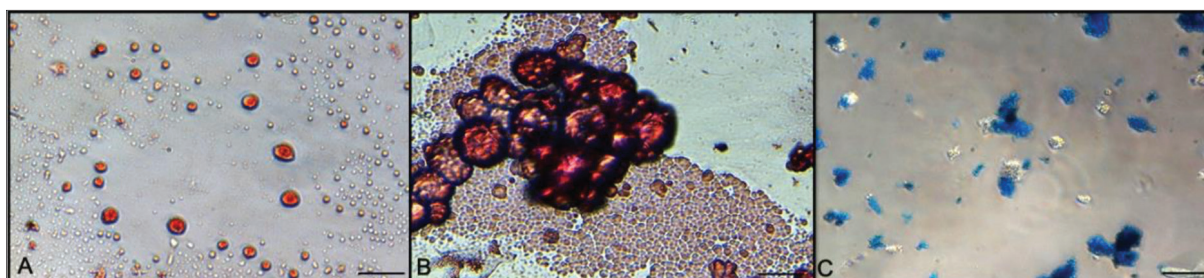
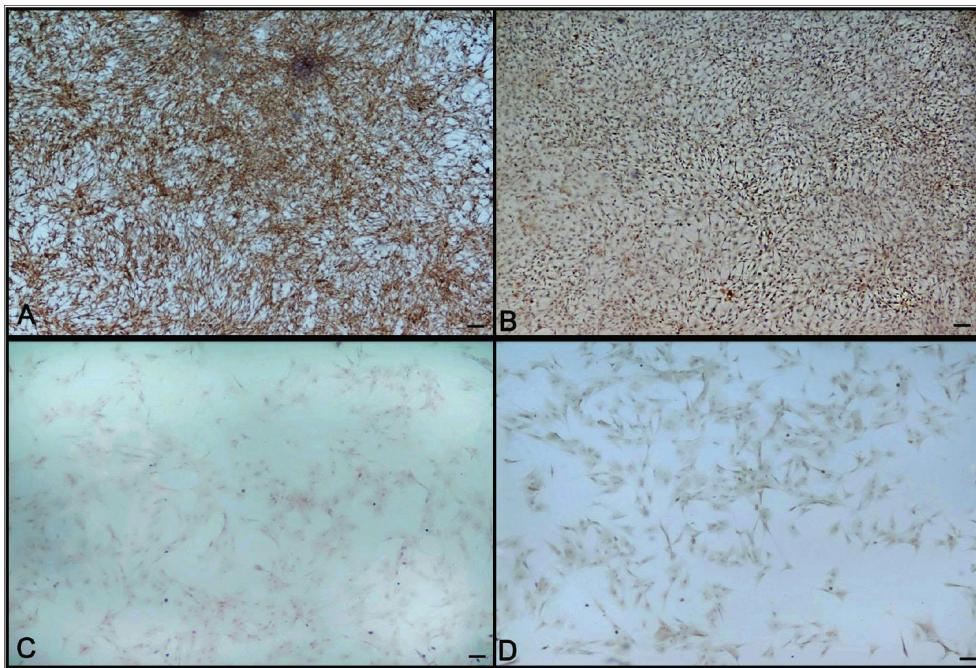
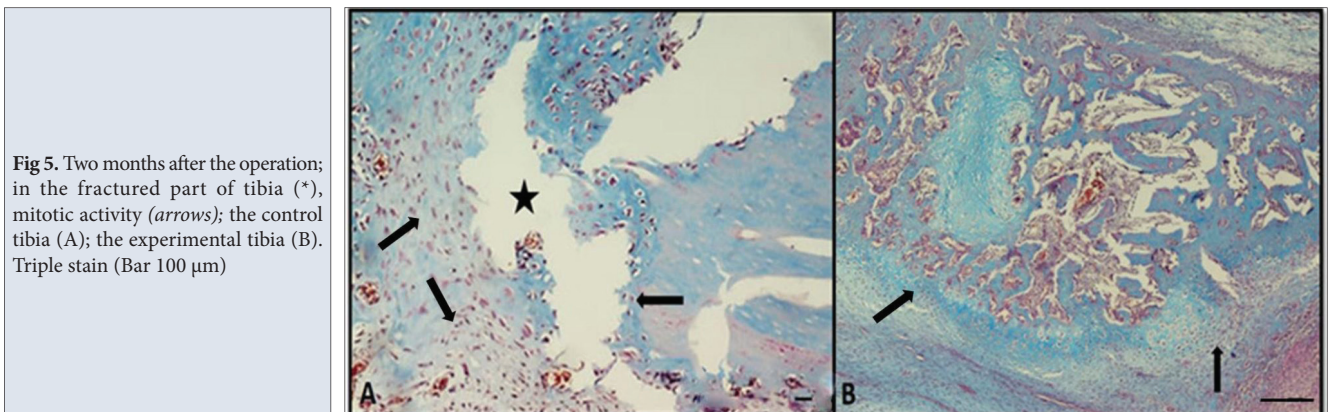


Fig 3. Adipogenic, osteogenic, chondrogenic differentiation of ADSCs were shown by Oil Red O (A), Alizarin red (B) and Alcian Blue (C) staining (Bar 25 µm)

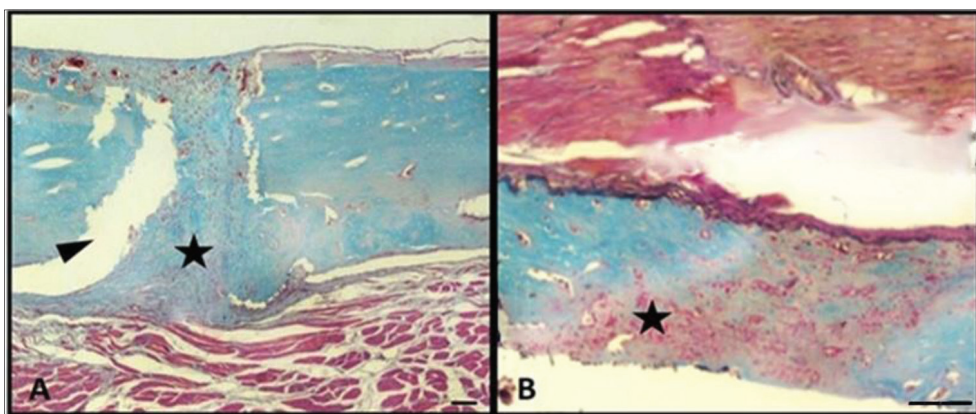




**Fig 4.** In P3 ADSCs: CD90 (A) and CD105 (B) were expressed positive reaction; CD 11b (C) and CD 45 (D) expressed negative reaction by immunohistochemistry (Bar 100 µm)



**Fig 5.** Two months after the operation; in the fractured part of tibia (\*), mitotic activity (arrows); the control tibia (A); the experimental tibia (B). Triple stain (Bar 100 µm)



**Fig 6.** At 60<sup>th</sup> day after the operation; the control tibia (A); the experimental tibia (B). Fractured part of tibia (arrow head) the matrix of decalcified bone of the experimental tibias (B) were showed strongly eosinophilic reaction (\*) than the control tibias. Triple stain (Bar 100 µm)

has been investigated histologically, clinically and radiologically in this study. ADSCs were preferred, because adipose tissue was a rich source of stem cells; thus, this study aimed to evaluate the osteogenic ability of grafted ADSCs on an absorbent material in rat tibias.

The distinct mechanisms of tissue regeneration mediated by stem cells are elucidated for many tissues [23,24]. Osteogenic regeneration depends on several factors including the biomaterial, bone quality, and surgical technique [25,26]. ADSCs and their extracellular secretions are major factors in osteogenesis; in this way, ADSCs represent an alternative

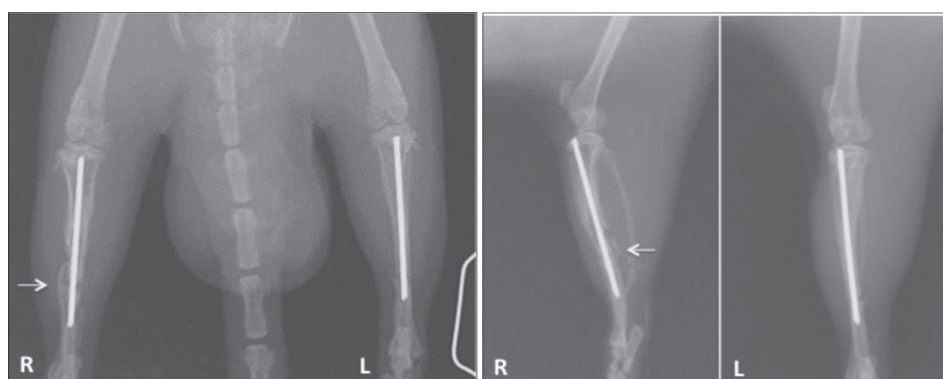


Fig 7. At 45<sup>th</sup> day after the operation, cranio-caudal and lateral radiographs of the tibiae in a rat, it may be clearly seen that there was callus formation in the caudal and lateral parts of the experimental (right) tibia (arrows) and bilateral visible fractured lines

source of osteogenic cell potential [27,28]. In the presented study, it has been showed that expression pattern of cell surface markers on P3 cells are associated with ADSCs. These markers (CD90, CD105, CD 11b, CD 45) have also been used to determine multi-lineage differentiation capabilities of ADSCs and examined P3 cells are determined by lipogenic, osteogenic and chondrogenic differentiation capability *in vitro* that detected using Oil Red O, Alizarin red and Alcian Blue staining, respectively. As a result of these findings, passage 3 cells ADSCs were safely used in the injected with an absorbent material. The osteo differentiation of P3 cells increases with induction by osteogenic culture medium. The capability of these cells to secrete mineralized extracellular matrix (ECM) was confirmed by the gradual increase in alizarin red staining (illustration of mineralized nodules secreted by differentiated cells).

Because of the role of MSCs in tissue engineering, ADSCs can be considered as a preferable cell source, as periosteal stem cells have a higher proliferation rate and osteogenic potential [29]. This also indicates that both BMSSCs and ADSCs are unable to differentiate into osteoblasts/odontoblasts *in vivo* early in the transplantation process, but can also induce host cells to participate in tissue regeneration with the formation of a hematopoietic marrow and a pulp. Connective tissue aids osteogenesis by stimulating blood vessel growth and hematopoietic marrow formation in BMSSC transplants [23]. In this presented study, new bone formations (callus) were investigated radiologically and histologically, and then the experimental group results evaluated significant differences regarding new bone formation as compared with the control group. At two months after postoperatively, there was no necrosis in both groups and there was granulation tissue. Moreover, osteoblastic cell proliferation and new bone formation (callus) were higher in the experimental group than in the control group. According to these results, it could be implied that adipose tissue-derived stem cells had potentiate osteogenesis.

Important role of the periosteum and endosteum is nutrition of bone cells and provision of osteoblasts for bone

histogenesis and repairmen [30]. These cells synthesize and secrete the osteoid matrix that is composed of glycoproteins and collagen. The secreted osteoid matrix has a high affinity for calcium salts that are brought into the area of bone formation by the circulatory system [31]. In this study, at two months after surgery the right (experimental) tibiae had more blood vessels than the left (control) tibiae. It was observed that the developmental difference in the ADSCs-treated groups sections were demonstrated with respect to the proliferation activity. The mitotic activity is prominent on the callus area of the right (experimental) tibiae. It has been shown that ADSCs also regulate osteogenic activity and bone regeneration. In this way, in the experimental tibiae, regeneration improved earlier compared with the control tibiae, and the bone regeneration by osteoblasts is observed at fractured part of the right tibiae. Furthermore, a strong eosinophilic reaction in the bone matrix of the right tibiae than left tibia was also detected. The presence of the eosinophilic mineralized bone matrix area was suggested the presence of bone histogenesis [32]. These results provide evidence that ADSCs was stimulated osteoblast activity and increases mineralized bone volume. Development of the new growth factors in ADSCs cultures would be induce superior osteogenesis [33]. ADSCs has also been found to act on chondrocytes and osteoblast stimulating their proliferation. The histologically and radiologically results of this study strongly support the mesenchymal stem cells obtained from adipose tissue of rats has been found to act on osteoblast activity and increases mineralized bone volume. In addition, allogeneic ADSCs to be an effective agent that can be used to increase bone regeneration and osteogenesis.

As a conclusion, it can be emphasized that ADSCs has superiority for osteogenesis and easily accessible therapeutic agent, and veterinary practitioners can be use ADSCs as a ready-to-use product to increase the bone healing follow up surgery.

#### AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current



study available from the corresponding author (T. İlhan) on reasonable request.

## FUNDING SUPPORT

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## CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

## ETHICAL STATEMENT

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (Approval no: 2018-02/03).

## AUTHOR CONTRIBUTIONS

Tİ, HS and HE planned, designed the experiment, analyzed all data and drafted manuscript. CÖA and Eİ collected samples, participated in cell culture analysis and collect data. Tİ, CÖA and Eİ responsible of histological examination and immunohistochemistry protocol. MÇ, UC and HS participated in surgical experimental protocol. All authors have read and agreed to the published version of the manuscript.

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