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# Effects of systemic erythropoietin treatment and heterogeneous xenograft in combination on bone regeneration of a critical-size defect in an experimental model

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ABSTRACT

The aim of the present study was to evaluate the effects of systemic EPO treatment alone or in combination with xenogenic bone graft augmentation on bone regeneration. Eleven adult male Sprague–Dawley rats were used in the present study. Rats were subjected to bilateral 5 mm critical size bone defects on the parietal bones under general anaesthesia. Right parietal bone defects were augmented with xenogenic bone graft and left parietal bone defect was left empty. Rats were randomly assigned for one of the two groups. One group of rats received (i) vehicle ($n = 6$) and other group received (ii) EPO (500IU kg/day) ($n = 5$). EPO treatment was continued for 28 days. Vascularization was analysed by immunohistochemical staining of CD31 (PECAM-1) and new bone formation was histomorphometrically evaluated. Xenogenic graft augmentation enhanced bone formation and vascularization significantly in either vehicle or EPO treated groups ($p < 0.05$). Histomorphometric results of angiogenesis was similar in the EPO treated group and the control group. However, angiogenesis was significantly higher in the combination of systemic EPO treatment with graft augmentation than graft augmentation alone ($p < 0.01$). Graft augmentation for treatment of critical size bone defects seems essential for proper bone healing. Results of the present study suggest that EPO potentiates the regenerative processes of augmented bone defects.



## 1. Introduction

Insufficient bone healing and quantity are consequences of tooth-related bone loss, such as traumatic tooth extraction, previous periodontal disease, or periapical pathology. Bone loss continues to be an important challenge for surgeons. A variety of materials and techniques were introduced and evaluated to enhance healing of bone defects. Biomaterials like autogenous, homogenous (allograft) and heterogeneous (xenograft) bone grafts, and synthetic (alloplastic) substitutes can be used with their osteogenic, osseoinductive and osseoconductive capacities to fill bone defects (Moore et al., 2001). Xenografts are obtained mainly from bovine bones, provide unlimited availability and, combined with proper processing minimize the risk of infection (Peres and Lamano, 2011). Osteoconductive characteristic of these graft materials provide microscopic and/or macroscopic scaffolding to aid bone regeneration by enhancing the internal migration of cellular elements involved in bone formation and angiogenesis (Dinopoulos et al., 2012). Local biochemical stimulation of graft materials with growth factors can be an advantageous alternative in bone healing by overcoming many limitations of the use of bone grafts alone (Peres and Lamano, 2011). Bone morphogenic protein-2 (BMP-2) and BMP-7 come into prominence with their osteoinductive property and clinical use among growth factors (Geiger et al., 2003; Dimitriou and Giannoudis, 2005). Other growth factors including platelet-derived growth factor, transforming growth factor- β, Insulin-like growth factor, vascular endothelial growth factor (VEGF) and fibroblast growth factor (Dimitriou et al., 2005), have

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been used with different functions in terms of cell proliferation, chemotaxis and angiogenesis, and are also being investigated or are currently being used to augment bone repair (Nauth et al., 2010).

In a physiologic manner osteoblastic cells provide their nutrition from vascular plexus of bone. Especially vascularity of tissue becomes more critical in the metabolically active regenerating callus. Besides providing nutrients and oxygen, angiogenesis support the growth factors, cytokines, mesenchymal cells and osteoblasts to regenerating tissues that are crucial for new bone formation (Eghbali-Fatourechi et al., 2005). Studies have shown that impaired bone vascularity results in inadequate osteogenesis in bone repair with decreased bone formation (Kleinheinz et al., 2005). Investigation of the importance of angiogenesis attracts researchers' attention to enhance angiogenesis for proper bone regeneration.

EPO is a physiologic hormone whose essential role is erythrocyte production. Therefore, EPO treatment has become the standard care for EPO deficient anaemia that occurs in most patients with chronic kidney disease, since the approval by the US FDA in 1989 (Hayat et al., 2008). Peritubular renal cortex is the main source of EPO in adult humans and EPO production is induced in the kidney by the hypoxia-induced transcription factor in response to reduced tissue $O_2$ pressure (Jones and Bergeron, 2001; Jelkmann, 2011). Besides EPO's physiologic effect of control over erythropoiesis, it also has non-hematopoietic effects (Mocini et al., 2007). Systemic EPO treatment increased expression of VEGF and VEGF mediated angiogenesis in the femoral segmental defects of mice (Holstein et al., 2011). It has been found that EPO enhanced chondrogenic and angiogenic responses during bone repair and may serve as a therapeutic agent to facilitate skeletal regeneration (Wan et al., 2014).

Besides its osteogenic and angiogenic effects of EPO in different bone defect models, little is known about potential regenerative effects of EPO on graft augmented defects. Osseoconductive effects of heterogeneous xenografts may potentiate regenerative effects of systemic EPO treatment and vice versa. The aim of this study was to assess formation of new bone and neovascularisation histomorphometrically after local xenograft augmentation and systemic EPO administration.

## 2. Materials & methods

### 2.1. Animals

Eleven 12 week old male Sprague–Dawley rats (270–300 g) were obtained from the experimental animal centre at Yeditepe University, Istanbul, Turkey. The study was approved by the Yeditepe University ethical Committee for Experimental Research on Animals. All animals were held under a constant 12:12-hr light/darkness regimen (lights on daily at 07.00 hr), where the temperature (22 ± 1 °C) and relative humidity (40–50%) were kept constant. The animals were maintained ad libitum with water and standard laboratory aliment. Efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Calvarial defect model

Prior to surgery, rats were anesthetized by intramuscular injection of a ketamine (60 mg/kg) and xylazine (10 mg/kg) combination. The rats' heads were stabilized with a stereotactic frame to prevent movement during surgical procedures. The surgical areas were shaved and the skin was disinfected with alcohol. A two cm length mid-sagittal skin incision was made. A periosteal incision and dissection was performed and care was taken to ensure that the periosteum was completely cleared from the surface of the cranium. Proper surgical access to parietal bones was provided with the help of four self-retaining ecartuers, these were made of insulin injector tips. 5-mm-diameter, critical size, circular bone defects were created with trephine burs with a low-speed handpiece under continuous saline irrigation to both parietal bones bilaterally symmetrical by the reference of the bregma point and sagittal suture. The full thickness of the cranial bone was removed and bone defects of right parietal bones were augmented with cortico-cancellous heterologous xenograft bone particles (M1005FE, Gen-Os, Osteobiol, Coazze, Italy) and bone defects of left parietal bones were left empty (Fig. 1-A). The periosteum was sutured to provide full periosteal coverage of bone defects and graft materials. Finally, the incision was closed with simple interrupted sutures.

### 2.3. Erythropoietin administration

Eleven rats were randomly divided into a control (n = 6) and EPO group (n = 5). Rats in the EPO group were treated by daily intraperitoneal injection of EPO (500U/kg body weight) (Neoreocormon, Novartis, Switzerland) dissolved in saline to reach a volume of 0.25 ml 0.25 ml saline without EPO was daily administered intraperitoneally to the control group for four weeks. With this method four different treatment modalities were applied (Fig. 1-B):

(1) No treatment (Control) (n = 5)
(2) Xenogenic graft augmentation (Graft) (n = 5)
(3) Systemic EPO treatment (EPO) (n = 6)
(4) Xenogenic graft augmentation and systemic EPO treatment (Graft + EPO) (n = 6)

### 2.4. Histomorphometric analysis

At the twenty eighth day following surgery, animals were sacrificed by an overdose of an anaesthetic agent. The calvaria of the rats were harvested for histomorphometric evaluation. Tissue

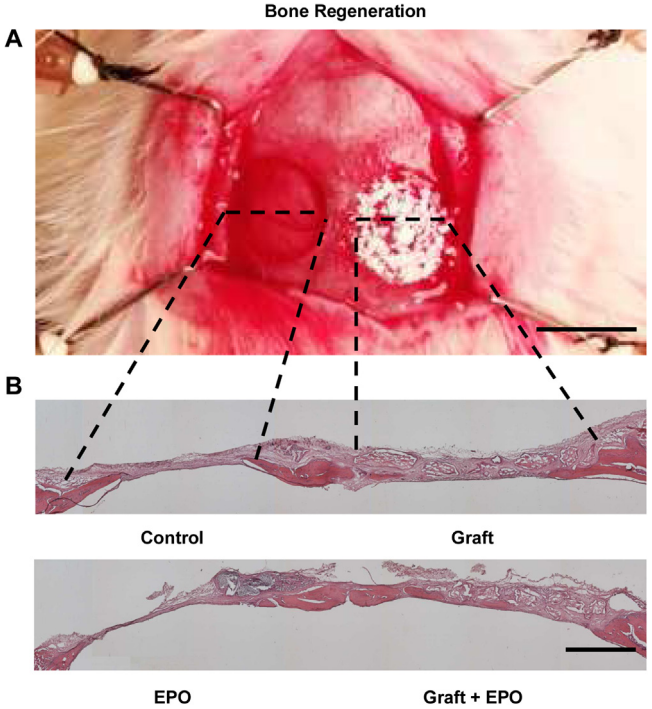


**Fig. 1. Evaluation of Bone Regeneration. A)** Representative photo of calvarial defects in both parietal bones. Right calvarial bone defects were augmented with cortico-cancellous heterologous xenograft bone particles (Bar = 5 mm). **B)** Evaluation of bone regeneration with end to end tissue sections of bone defects (Staining: Hematoxylin & Eosin, Bar = 2 mm).

samples were fixed in 10% neutral formaldehyde in 0.1 mol of phosphate buffered saline solution (pH, 7.4). Samples were decalcified with Morse solution (10% sodium citrate and 22.5 % formic acid) that was replaced twice a week for 4–5 weeks. Samples were rinsed in tap water for 12 h, dehydrated with 30% sucrose overnight and embedded to tissue freezing mediums (Tissue-Tek, Sakura Finetek, Breisgan, Germany) for cryosectioning. Serial sections were cut at 10 μm in the coronal plane to examine bone defects from end to end (Fig. 1-B). Every fiftieth section was selected through a set of consecutive sections for histomorphometric investigation. The tissue sections were mounted on positive charged slides. A total of nine sections were investigated for each tissue sample. During serial sectioning the investigator paid attention to remove tissue sections from the same areas of the different defects. Serial tissue sections were stained with haematoxylin-eosin for investigation of new bone formation. Angiogenesis was analysed by staining of CD31 (Fig. 2-A). Images of sections were analysed using a microscope (Axio Zoom V16, Carl Zeiss, Göttingen, Germany). Histomorphometric analysis was performed by summation of newly formed bone areas and vessel areas in the serial sections of the tissue samples.

### 2.5. Immunohistochemical analysis

Tissue sections were pre-treated for antigen retrieval with 0.01 M citrate buffer (pH 5.0), rinsed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton X-100 and 10% normal donkey serum. Sections were incubated overnight at 4 °C with monoclonal rat anti-CD31 (1:1000, #557355; BD Biosciences) antibody and detected with Cy3-or Cy2-conjugated secondary antibodies. Sections were analysed using a confocal microscope (LSM 780, Carl Zeiss, Göttingen, Germany).

### 2.6. Statistical analysis

All present data are given as means ± standard deviation. Comparison between the experimental groups was performed by one-way analysis of variance (ANOVA). Post hoc analyses using LSD test were performed to detect pairs of groups with statistical differences. The data were analysed using the SPSS PASW Statistics 18.0. A $p$-value <0.05 was considered to indicate significant differences between experimental groups. Sample size, bone formation results and significance level of the present study provided 94% power according to results of the post-hoc power analysis.

## 3. Results

### 3.1. Bone formation

Bone apposition was noticeable on the surface of the graft particles, and new bone formation was centripetal from the host bone in the margin of the defects. Bone formation in the non-grafted samples was in the form of bone islands and had immature characteristics with the presence of surrounding cuboidal osteoblasts.

Histomorphometric analysis of new bone formation revealed that bone formation of the graft group was significantly higher than the control (p = 0.036) group. New bone formation was significantly greater in the graft + EPO group when compared to the control (p = 0.001) and EPO groups (p = 0.004). However, new bone formation in bone defects showed no difference between the graft + EPO group and the graft group. The comparison between the treatments revealed no significant differences between the EPO and the control group (p = 0.617) (Fig. 2-B).

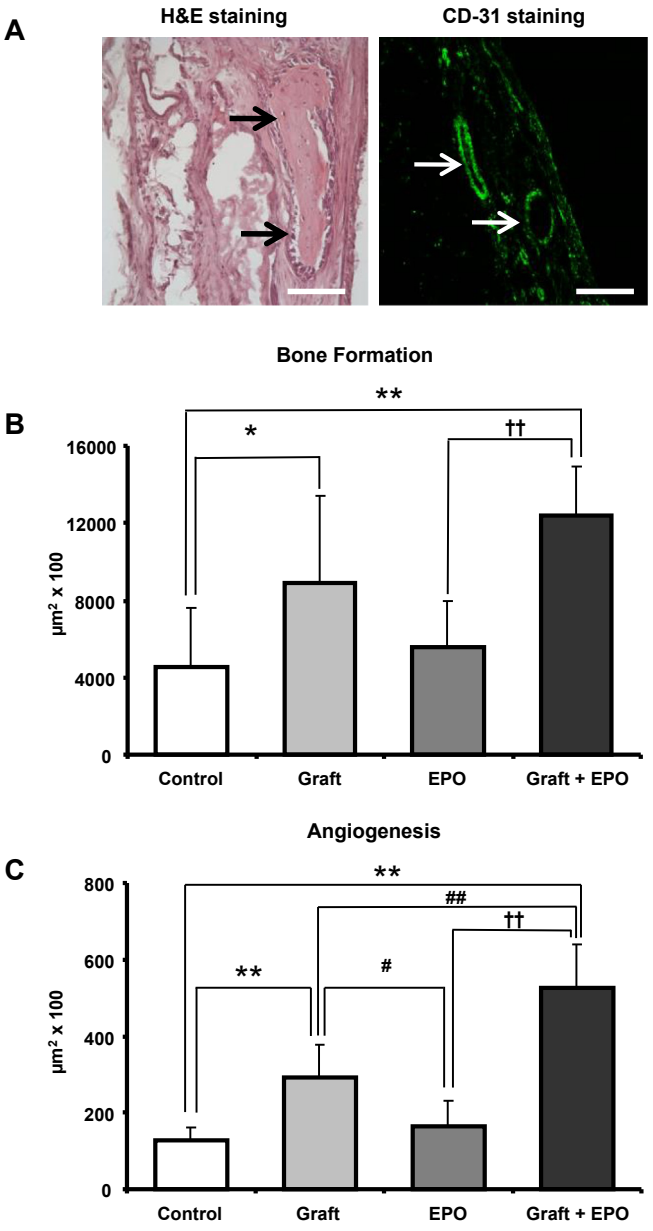


**Fig. 2. A)** Representative images of Hematoxylin &Eosin staining for evaluation of bone formation and CD 31 immunohistochemical staining for evaluation of angiogenesis (Bars = 100 μm). **B)** New bone formation and **C)** angiogenesis was assessed using calvarial defect sections. Histomorphometric analysis of new bone formation revealed that bone formation of graft group (p = 0.036) and graft + EPO (p = 0.001) group was significantly higher than the control group. New bone formation was greater in graft + EPO group compared to EPO group (p = 0.004). Quantitative assessment of the angiogenesis revealed a significantly higher vessel density in the graft group (p = 0.002) and graft + EPO (p = 0.001) group compared to the control group. Vessel density in the EPO treated group did not differ significantly from that in vehicle treated control group (p = 0.415). However, significantly higher vessel density was observed in the graft + EPO group compared with the graft group (p = 0.001). Notably, effect of systemic EPO treatment on angiogenesis was even more pronounced in graft augmented bone defects compared to non-augmented bone defects. In addition to that higher vessel density noted in the graft group (p = 0.014) and graft + EPO (p = 0.001) group compared to the EPO group. *$p < 0.05$/**$p < 0.01$ compared with vehicle-treated control group. #$p < 0.05$/##$p < 0.01$ compared with graft augmented group. †$p < 0.05$/††$p < 0.01$ compared with EPO-treated group. Values are given as mean ± SD.

### 3.2. Angiogenesis

Quantitative assessment of the angiogenesis within the bone defects revealed a significantly higher vessel density in the graft

group compared to the control group ($p = 0.002$) and the EPO group ($p = 0.014$). Vessel density in the EPO treated group did not differ significantly from that in the vehicle treated controls ($p = 0.415$). However, significantly higher vessel density was observed in the graft + EPO group compared with the graft group ($p = 0.001$) (Fig. 2-C).

## 4. Discussion

Proper bone healing necessitates close spatial and temporal coordination of molecular and cellular processes involving resident bone cells, inflammatory cells and associated vascular structures. This complex physiological process of bone regeneration includes the utilisation of growth factors, osteoconductive scaffolds, mechanical environment and osteogenic cells, also known as the triangular diamond concept (Seebach et al., 2010). Therefore, local biochemical stimulation of graft materials with growth factors was recommended as an advantageous alternative in procedures aimed at stimulating bone healing (Peres and Lamano, 2011). In this research we hypothesized that local xenograft augmentation and systemic EPO administration may have a cumulative healing effect. Bilateral bone defects were created on the calvarium of rats and right bone defects were augmented with xenograft and one group received systemic EPO treatment while the other group received vehicle for a period of four weeks. Histomorphometric investigation of new bone formation and angiogenesis were performed. The results indicated that local xenograft augmentation enhanced new bone formation and angiogenesis in both the EPO treated and vehicle treated groups. However, systemic EPO treatment had no influence on new bone formation or angiogenesis in the non-augmented calvarial bone defects. On the other hand, notable differences in angiogenesis between graft group samples and graft + EPO group samples indicated that systemic EPO treatment enhanced angiogenesis in the xenograft augmented calvarial bone defects.

The experimental study of Sun et al. revealed that daily subcutaneous injection of EPO to the calvarial defect area had no influence on bone formation. However, daily subcutaneous injection of EPO enhanced angiogenesis and bone quality when combined with BMP-2 enriched gelatine scaffolds (Sun et al., 2012). Nair et al. showed that microbubble scaffolds loaded with EPO significantly increased bone volume in the calvarial defect compared to unloaded microbubble scaffold (Nair et al., 2013). In contrast, local application of EPO to different scaffolds or autograft did not influence the results, however augmentation of autogenous graft significantly enhanced bone healing in the calvarial defects of pigs in a previous study (Rölfing et al., 2014). Differences between studies can be attributed to the different scaffold properties such as scaffold architecture, mechanical properties and manufacturing technology which is a determinant factor for success of the scaffold (O'Brien, 2011). In the present study, histomorphometric analyses showed that xenograft enhanced bone formation while systemic EPO had no effect on bone formation in the calvarial defect at the end of 4 weeks of healing. Xenografts materials are inert osteoconductive filler materials. Osteoconduction is the graft's ability, due to its microscopic and/or macroscopic scaffolding to aid the healing process by enhancing the internal migration cellular elements involved in bone formation (Dinopoulos et al., 2012). In the present study xenograft augmentation significantly enhanced bone formation in the calvarial defects of both vehicle treated and EPO treated animals. Results were consistent with previous research and confirmed enhanced bone healing via augmentation of xenograft materials (Tapety et al., 2004; Bosco et al., 2016).

Potential angiogenic effects of EPO were evaluated with femoral segmental defect and an increased number of blood vessels per unit of assessed area at 2 weeks was noted while the blood vessel density did not differ between EPO-treated mice and vehicle-treated mice at 10 weeks (Holstein et al., 2011). Enhancement of new bone formation and neovascularisation is attributed to the increase of VEGF in the early phase of healing that has both osteogenic and angiogenic capacities. To the best of our knowledge this is the only study that investigated both early and late phases of bone healing after EPO treatment by histomorphometry. In another study, vessel density was analysed in periosteal, central and endosteal zones of the callus after 2 weeks of healing in a mouse femoral osteotomy gap. Quantitative analysis of vessel density was significantly higher in the endosteal healing zone that was not significantly different in the periosteal and the central part of the healing zones. However, this study only provides results for the early healing period (Garcia et al., 2011). According to the study of Rölfing et al. the number of blood vessels was similar in the EPO-treated collagen carrier and saline treated collagen carrier group at the end of 5 weeks of healing that used the porcine calvarial defect model (Rölfing et al., 2014). In the present study, systemic EPO treatment did not significantly increase angiogenesis from that in the vehicle treated controls at the end of the 4 weeks healing period in the calvarial defect model. However, the intrinsic differences between animal species may lead to different regeneration speed and capacity. Therefore, comparing the results of different animal models may give rise to misleading conclusions. Within the limited available knowledge about histomorphometric consequences of the angiogenic effects of systemic EPO treatment, systemic EPO treatment seems to enhance angiogenesis in the early healing period of bone healing. In contrast to enhanced molecular regenerative parameters of the previous research, histomorphometric consequences of EPO treatment have controversial results and need further investigation.

Vascularization of bone graft materials is an important process for prompt and long term successful osteogenesis. In addition, the scale of angiogenesis is related to the stimuli present in the surrounding tissues that allow pre-existing vessels to commence budding into the freely applied grafts and graft material itself (Dinopoulos et al., 2012). Due to the porosity of xenografts not only infiltration by bone forming cells but also infiltration of growth factors were allowed (Dinopoulos et al., 2012). So stimulation of angiogenesis in the surrounding tissues may accelerate osteogenesis and long term success. Potential angiogenic effects in the xenograft augmented and non-augmented defects were investigated in the present study. Xenograft augmentation significantly enhanced angiogenesis in the calvarial defects of both vehicle treated and EPO treated animals. In addition, a significant difference between the graft and graft + EPO group was noted while there was no significant difference between the control and EPO group. There were considerable differences between the graft and graft + EPO groups, while a lack of differences between the control and EPO is remarkable. Xenografts provide scaffolding for vascular formations and growth factors, and therefore seem to be able to potentiate the angiogenic effect of EPO during the healing process of calvarial bone defects.

Translation of these experimental studies into clinical trials requires a physiological dosage of EPO in order to avoid complications, such as thrombo-embolism, cerebral convulsion/hypertensive encephalopathy and arterial hypertension (Singbartl, 1994). Repetitive EPO injections ranging from 500 to 5000 IU/kg were administered in different in vivo studies (Holstein et al., 2007; Garcia et al., 2011) which have a systemic effect and potential risk of adverse events. Therefore, testing of the effective and clinically safe dose of EPO is essential before clinical trials can be considered. Garcia et al. evaluated daily systemic treatment of low dose (500 U/kg) EPO. They found that low dose EPO increased biomechanical

stiffness and radiological density of the femoral osteotomy gap. They noted significantly greater haemoglobin concentrations in blood samples from EPO-treated animals without any observed side effect (Garcia et al., 2011). In another study of Holstein et al. they applied a high dose of EPO (5000 U/kg) for a short time (6 days) and stated that treatment enhanced early endochondral ossification and mechanical strength in closed femoral fracture model in mice. 5000 U/kg EPO-treatment resulted in a slight, but not a significant elevation of the haemoglobin concentration after 6 days of treatment and no adverse effect was noted (Holstein et al., 2007). The rather low dosage of 500U/kg was chosen in the present study in order to minimize the potential side effects of EPO with no side effects noted in the experimental group. The only clinical study about EPO on bone regeneration investigated the effects of local EPO injection in healing of tibiofibular fractures and stated that no patient experienced any adverse effect (Bakhshi et al., 2012). Unfortunately, it is impossible to conclude about effective systemic dosage of a drug from results of a research that investigated effects of local injection. Further clinical studies are needed to identify the minimum effective dose for EPO.

EPO treatment influences the cellular mechanisms during regeneration of bone tissue, and in the present study, histomorphometric examination was used to determine the effects of EPO treatment. However, more detailed studies on the effects of EPO during regeneration of bone defects should be explored with more sophisticated investigation methods. In addition, there are contradictory results about consequences of EPO treatment with different application methods and on different types of bone defects during the short and long term healing period. Future studies should also target the effective dosage and route of the EPO treatment.

## 5. Conclusion

Within the limitations of the present study, it can be concluded that systemic EPO has no effect on angiogenesis and bone formation of critical-size calvarial bone defects at the end of four weeks. Xenograft augmentation for the treatment of bone defects enhances both angiogenesis and bone formation essential for the physiological function of bone. The present findings corroborate the idea that critical size bone defects require a graft for proper bone healing. Furthermore, the present study indicates that xenograft augmentation potentiates the angiogenic effect of the EPO treatment and systemic EPO treatment may be a promising agent for adjuvant therapy during xenograft augmented bone healing.

## Ethical statement

The study was approved by the Yeditepe University ethical Committee for Experimental Research on Animals.

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## Declaration of interest

None.

The authors declare no financial or other conflict of interest concerning the present study.

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