Effect of Graft Application and Nebivolol Treatment on Tibial Bone Defect in Rats

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OBJECTIVE: To evaluate the results of the effect of nebivolol on tibial bone defect and graft application in new bone development in the rat.
STUDY DESIGN: Thirty Wistar albino rats were divided into 3 groups. In the Control group, tibia bone defect was created without any treatment. In the Defect+Graft group, allograft treatment was performed by forming a 6 mm tibial bone defect. In the Defect+Graft+Nebivolol group, alloplastic bone graft was placed in the calvarial bone defect and then nebivolol (0.34 mg/mL solution/day) treatment was intraperitoneally applied for 28 days.
RESULTS: Histopathological examination revealed inflammation in the defect area, congestion in the vessels, degeneration in collagen fibers, and an increase in osteoclast cells. There was an increase in inflammation and blood vessel structure in graft application, and osteoblastic activity matrix formation after reorganization nebivolol application in collagen fibers. Osteonectin expression was positive in the collagen fiber and matrix, starting in the Graft group, in osteoblasts, whereas in the Nebivolol group, osteoblasts increased in osteocytes and new bone formation.
CONCLUSION: Nebivolol is thought to have a positive effect on osteoinductive bone growth factors and contribute to the cell-matrix interaction, in addition to the supporting effect of the graft with its antioxidative effect. (Anal Quant Cytopathol Histpathol 2021;43: 223–228)

Keywords: allograft; bone; bone regeneration; disease models, animal; nebivolol; orthopedic procedures; osteonectin; rats; tibia; tibial defect.

Repair of critical bone defects is among the major challenges in orthopedic practice. Among methods of treating bone defects, bone autograft, allograft, xenograft, and implantation of artificial bone are important.[1] Limitations of bone autograft are insufficient material for transplantation and possible complications at donor sites. Deficiencies of allograft and xenograft constitute graft rejection, inadequate repair and transplantation, and potential infections in remote areas.[2] The use of bone graft in orthopedic surgical methods has an important place in increasing bone regeneration. Osteoconduction, osteoinduction, and osteogenesis formation are important processes in bone regeneration.[3] Autograft is a widely used bone graft due to its osteoinductive and osteoconductive properties. Since allografts have an osteoinductive effect in the areas where they are applied, if the vascular

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Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

0884-6812/21/4304-0223/$18.00/0 © Science Printers and Publishers, Inc.

structures in the microenvironment respond well, they give good results as expected from an autogenous graft. Allograft is used as a bone replacement because it prevents donor site morbidity.[4] During bone formation, extracellular matrix (ECM) and organic and inorganic compounds have a significant effect. There are many factors involved in bone formation, including fibronectin, matrix metalloprotein family osteoclast markers, osteopontin, and osteonectin.[5] Osteonectin is synthesized by cells of the osteoblastic lineage; binds hydroxyapatite, calcium, and type I collagen; and inhibits mineralization in vitro.[6]

Nebivolol is a relatively new highly cardio selective β-adrenergic receptor antagonist that possesses endothelium dependent vasodilator properties and antioxidant capacities.[7] Nebivolol also exerts antioxidant effects by stimulating nitric oxide synthesis. Studies have shown that a dose of 5 mg effectively lowers blood pressure over a 24-hour period and causes endothelium-dependent vasodilation.[8]

The aim of this study was to evaluate the results of the effect of nebivolol on tibial bone defect and graft application in new bone development in the rat.

Materials and Methods

The study protocol was approved by the Animal Research Committee of Dicle University, Turkey. Thirty adult Wistar albino rats, each weighing 250–280 (±10) g, were used as experimental animals. The animals were fed *ad libitum* water and standard laboratory animal diet. The rats were divided into 3 groups as follows: Control group (Defect group): tibial bone defect was created on the first day of the study and rats were kept immobile for 28 days. Defect+Graft group: a 6 mm tibial bone defect with allograft treatment was applied on the first day of the study and rats were kept immobile for 28 days. Defect+Graft+Nebivolol group: after exposing the right proximal tibia of each animal, a standardized 6.0 mm diameter noncritical bone defect was created by using a motorized drill under irrigation with saline solution.[9] An alloplastic bone graft was placed in the tibial bone defect on the first day of the study and nebivolol treatment was applied. Nebivolol was prepared from 5 mg of nebivolol tablets (Vasoxen, Menarini Group, I. E. Ulagay, Germany) by pulverizing and dissolving in distilled water to obtain 0.017 mg/mL solution.[10] Sterile nebivolol solution was administered intraperitoneally with 2 mL every day for 4 weeks.

At the end of the study, animals were sacrified by decapitation. The skin, as well as all of the soft tissues surrounding the tibia bone, were removed. The samples were fixed with 10% neutral buffered formalin solution and decalcified with 5% ethylenediaminetetraacetic acid (EDTA). After rinsing with tap water, the samples were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Tissue sections of 5 μm thickness were prepared in the transverse plane and stained using Masson's trichrome for light microscopy examination.

Immunohistochemical Analysis

Antigen retrieval was done in a microwave (Bosch, 700 watt) for 2 minutes at 90°C. They were subjected to a heating process in a microwave oven at 700 watts in a citrate buffer (pH 6) solution for proteolysis. Sections were washed in 3×6 min PBS and incubated with hydrogen peroxide (K-40677109, 64271 hydrogen peroxide [H_2O_2], Merck, Dortmund, Germany) (3 mL 30% H_2O_2+27 mL methanol) for 20 minutes. Sections were washed in 3×5 min PBS min and blocked with Ultra V Block (lot: PHL150128, Thermo Fisher, Fremont, California, USA) for 8 minutes. After draining, primary antibodies were directly applied to sections distinctly osteonectin (SPARC, catalog #33-5500, Thermo Fisher). Sections were incubated and left overnight at 4°C. Sections were washed in 3×5 min PBS and then incubated with biotinylated secondary antibody (lot: PHL150128, Thermo Fisher) for 14 minutes. After washing with PBS, streptavidin peroxidase (lot: PHL150128, Thermo Fisher) was applied to sections for 20 minutes. Sections were washed in 3×5 min PBS, and DAB (lot: HD36221, Thermo Fisher) was applied to sections up to 15 minutes. Slides showing reaction was stopped in PBS. Counterstaining was done with Harris's hematoxylin for 45 seconds, dehydrated through ascending alcohol series, and cleared in xylene (product no. HHS32, Sigma, hematoxylin solution, Harris Modified, Sigma-Aldrich, St. Louis, Missouri, USA). Slides were mounted with Entellan (lot: 107961, Sigma-Aldrich) and examined under light microscope (Zeiss, Germany).[11]

Statistical Analysis

The data obtained in the study were expressed as arithmetic mean±standard deviation. Statisti-

cal analyses were made using the SPSS program. In comparison of the groups, Kruskal-Wallis test and Bonferroni corrected post-hoc test were used. P<0.05 was taken as the significance level.

Results

All morphological changes in congestion of blood vessels, inflammation, and new bone formation were noted in detail. The intensity of these changes was graded from 0 to 4.[12]

Histopathological and osteonectin expression parameters of all groups are shown in Table I. Inflammation and congestion in blood vessels were increased significantly in the Defect and Defect+Graft groups (groups 1 and 2) as compared to the Defect+Graft+Nebivolol group (group 3) (p=0). For the new bone formation, a significant increase was observed in the Defect+Graft+Nebivolol group as compared to the Defect and Defect+Graft groups (p=0) (Figure 1).

Osteoblast and osteocyte cell activity were weak in the Defect group. However, osteoblast and osteocyte activity in the Graft and Nebivolol groups were statistically more significant (Table I) (Figure 2). However, while osteoclast cells increased in the Defect group, the experimental groups decreased (Table I) (Figure 2).

On histopathological examination in the Defect group, dilation and congestion in blood vessels, degenerative changes in collagen fibers, hyperplasia in osteoblast cells, hypertrophy in osteoclast cells, and an increase in the number of osteoclast cells were observed with increased inflammation in the defect area. In the Defect+Graft group there was a decrease in inflammation and mild dilation in blood vessels, and reorganization due to increase in synthesis of collagen fibers. It was observed that osteoblastic activity increased and osteoclast cells decreased. In the Defect+Graft+Nebivolol group it was observed that osteoblastic activity was intense, osteocyte cells increased, and the matrix structure was dense.

In the Defect group, osteonectin positive expression was observed in osteoclast cells and degenerative collagen fibers and a small number of osteoblast cells. In the Defect+Graft group, positive osteonectin expression was observed in osteoblast cells in the region close to the graft area, in some osteoclast cells, and in osteocyte cells in new bone trabeculae where matrix development has started. In the Nebivolol group, an increase in osteonectin expression was observed in the osteoinductive cells and newly formed osteocyte cells in the bone matrix.

Table I *Immunohistochemical and Histopathologic Score Values*

Parameter	Group	N	Mean±SD	Mean rank	Kruskal-Wallis test value	Multiple comparisons for groups (p<0.05)
Osteoblast osteonectin expression	(1) Defect	8	0.65±0.42	4.12	17.882	(2)(3)
	(2) Defect+Graft	8	2.52±0.61	13.64	p=0	(1)
	(3) Defect+Graft+Nebivolol	8	3.32±0.74	17.22		(1)
Osteocyte osteonectin expression	(1) Defect	8	1.08±0.42	5.24	18.122	(3)
	(2) Defect+Graft	8	2.29±0.54	12.64	p=0	
	(3) Defect+Graft+Nebivolol	8	3.52±0.58	19.78		(1)
Osteoclast osteonectin expression	(1) Defect	8	3.28±0.48	19.92	17.963	(2)(3)
	(2) Defect+Graft	8	1.56±0.68	10.88	p=0	(1)
	(3) Defect+Graft+Nebivolol	8	0.72±0.44	5.92		(1)
Inflammation	(1) Defect	8	3.22±0.68	18.16	17.124	(3)
	(2) Defect+Graft	8	2.12±0.48	14.86	p=0	(3)
	(3) Defect+Graft+Nebivolol	8	1.02±0.49	4.88		(1)(2)
Congestion blood vessels	(1) Defect	8	3.26±0.54	18.50	17.228	(3)
	(2) Defect+Graft	8	2.72±0.74	14.38	p=0	(3)
	(3) Defect+Graft+Nebivolol	8	1.34±0.46	4.44		(1)(2)
New formation	(1) Defect	8	0.98±0.76	5.48	15.884	(3)
	(2) Defect+Graft	8	2.12±0.72	12.22	p=0	
	(3) Defect+Graft+Nebivolol	8	2.98±0.52	18.46		(1)

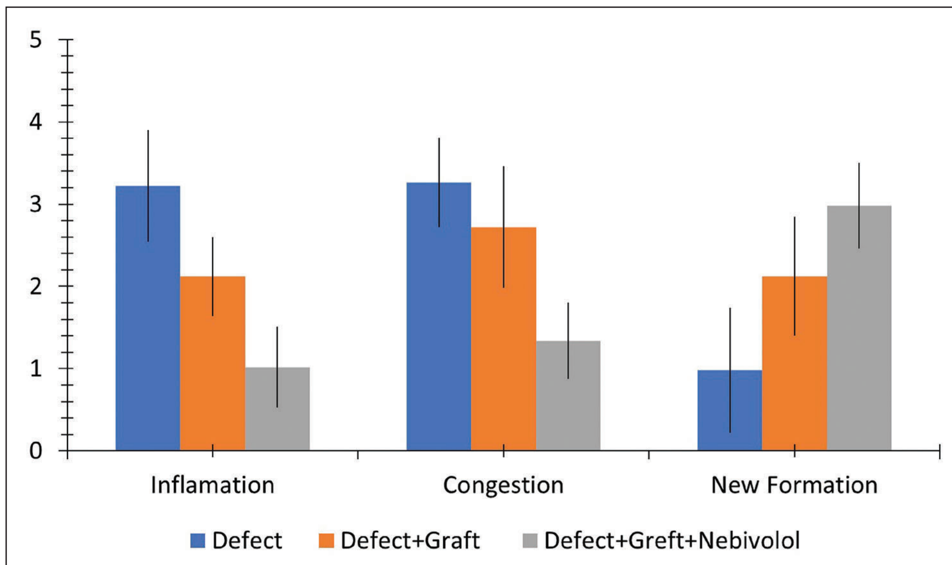

Figure 1
Histogram showing statistical analysis of histological parameters in all groups. The quantification of all parameters: 0 = no change, 1 = too weak, 2 = weak, 3 = middle, 4 = strong. Scoring was determined by examining histological parameters in 20 different regions within the microscope field.

Discussion

Regeneration of bone occurs after infiltration of granulation tissue, remodeling of osteogenic cells with proliferation. A cellular activity initiated by an acute inflammatory response and bone formation occurs through the association of specific cell types such as mesenchymal stem cells and various factors such as osteoclasts, hydroxyapatite, extracellular matrix molecules, cytokines, and bone morphogenetic proteins, hormones, vitamins, and growth factors.[13] Soft tissue trauma such as impaired blood circulation and inflammation as a result of loss of connections at proximal and distal points in regional fractures increases the risk of fracture healing.[14] Monfoulet et al[15] examined the kinetics and healing model of bone lesions in mice in two different structures, consisting of holes in the femoral region and the distal epiphysis. The healing of the epiphyseal defect occurred after only 13 weeks. The recovery in bone damage de-

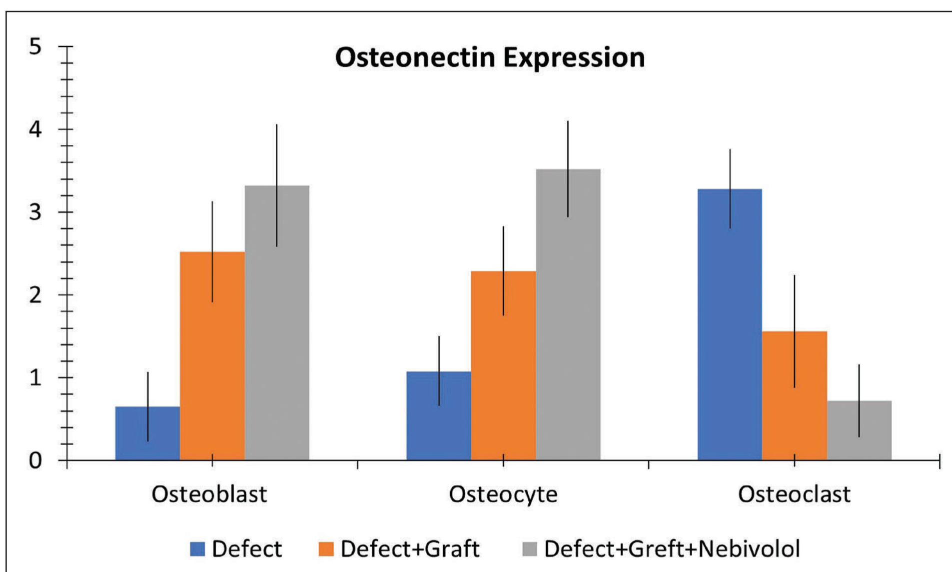

Figure 2
Immunohistogram showing statistical analysis of osteonectin expression parameters in all groups. The quantification of all parameters: 0 = no change, 1 = too weak, 2 = weak, 3 = middle, 4 = strong. Scoring was determined by examining histological parameters in 20 different regions within the microscope field.

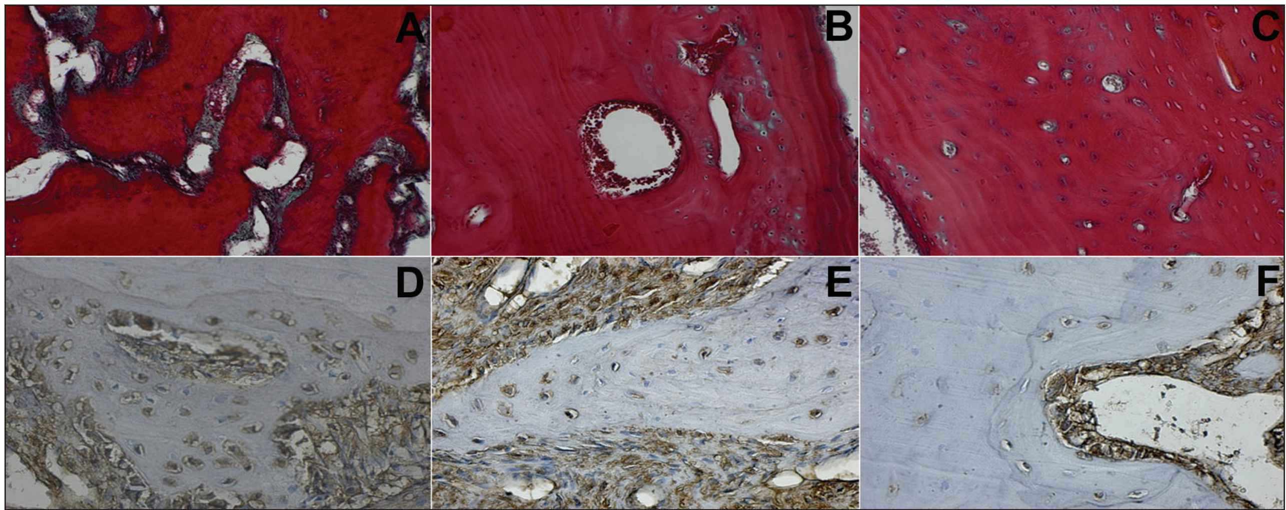

Figure 3 (A) Defect group. Dilation and congestion in blood vessels, degenerative changes in collagen fibers, hyperplasia in osteoblast cells, hypertrophy in osteoclast cells, and an increase in the number of osteoclast cells in the defect area (Masson's trichrome staining, bar = 100 μm). (B) Defect+Graft group. A decrease in inflammation and mild dilation in blood vessels. An increase in synthesis of collagen fibers (Masson's trichrome staining, bar = 100 μm). (C) Defect+Graft+Nebivolol group. An increase in osteoblastic activity, osteocyte cells, and development in the matrix structure (Masson's trichrome staining, bar = 100 μm). (D) Defect group. Positive osteonectin expression in osteoblast cells in the endosteum region near the bone marrow and collagen fibers within the osteon area (osteonectin immunostaining, bar = 100 μm). (E) Defect+Graft group. Osteonectin expression in the collagen fibers, osteoclast cells, and hyperplastic osteoblast cells around the blood vessels, decrease in osteonectin expression within trabecular bone (osteonectin immunostaining, bar = 100 μm). (F) Defect+Graft+Nebivolol group. Positive osteonectin expression in osteoblast cells and osteocyte cells in the periphery of wide bone trabecular (osteonectin immunostaining, bar = 100 μm).

pends on the defect morphology and the graft material, and grafts with the potential to have an osteoprogenitor effect in bone repair or regeneration are used. Bone regeneration was investigated *in vivo* by creating critical bone defects in the parietal bones of aging female rats. Serum albumin-coated bone allograft (bone albumin) has been reported to increase osteoblastic activity at the defect site and in aging rats and induce faster bone formation.[16]

Laçin et al[17] reported that the effects of allopurinol treatment on rat calvaria defects, when used in conjunction with alloplastic graft, may promote osteoblastic activity, matrix development, mature bone cell formation, and new bone development. Yiğit and Deveci[18] showed that allograft material stimulates the osteoinduction phase, causes the formation of osteoprogenitor cells, the initiation of osteoblastic activity, reduction of inflammation, and necrotic reduction. In our study, histopathological changes such as increased inflammation in the defect area, degenerative changes in collagen fibers, hyperplasia in osteoblast cells, hypertrophy in osteoclast cells, and an increase in the number of osteoclast cells were observed over 4 weeks (Figure 3A). After the graft application, the reduction of dilation and inflammation in blood vessels, reconstruction due to the increase in the synthesis of collagen fibers, development of osteoblastic activity, and reduction of osteoclast cells were evaluated as a positive result in the direction of recovery (Figure 3B). Osteoblastic activity is more intense, osteocyte cells increase and matrix structure development after the application of Nebivolol with antioxidative effect is considered as a positive indicator of new bone formation (Figure 3B).

Osteonectin is a glycoprotein involved in the mineralization of bone and cartilage matrices, cell-matrix interactions, and collagen binding in osteoblasts and odontoblasts. Osteonectin increases the production and activity of matrix metalloproteinases used as an indicator of new bone formation.[15] In a study, they observed that in ovariectomized rats, there was an acceleration in the formation of new bone with the osteoinductive effect, which increased osteoblastic activity after tamoxifen application, in which the osteoblast and osteoclast cells in the collagen fibers in the tibial region

decreased.[19] In this case, osteonectin can support bone by promoting mineral accumulation and regulating the growth and proliferation of mineral crystals. Mice with osteonectin deficiency develop poor bone condition and develop osteopenia with significant trabecular bone loss.[20] Koparal et al[21] have shown that matrix synthesis and mineralization increase and osteonectin expression increases under the effect of melatonin against the damage after tibial defect. In our study, osteonectin expression was positive in osteoclast cells and degenerative collagen fibers and a small number of osteoblast cells within the defect area, and increased osteonectin expression was observed in osteoblast cells due to the graft effect in the Defect+Graft group. Osteocyte cells in new bone trabeculae where matrix development begins with the reduction of graft inductive effect in nebivolol application. An increase in osteonectin expression was observed. As the allograft material decreases the callus development and increases the collagen activity in the connective tissue, the osteoinductive effect of the osteo-inductive cells leads to the development of invasive osteoblast cells, leading to the development of matrix and osteoblast cells in the formation of new bone.

Conclusion

Considering that osteonectin, a calcium-binding protein, plays a role in cell adhesion, it is important to increase the expression of osteonectin in osteoblast and later osteocyte cells.

Nebivolol is thought to have a positive effect on osteoinductive bone growth factors and contribute to the cell-matrix interaction, in addition to the supporting effect of the graft with its antioxidative effect.

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