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Original research

EFFECT OF TIDEGLUSIB WITH BONE GRAFTING ON NEW BONE FORMATION

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ABSTRACT

Objectives: The goal of this study was to observe the regenerative potential of Tideglusib in combination with autogenous and xenograft mandibular defects in rats.

Material Methods: Our study consists of five groups: one control and four experimental. In 40 Wistar albino rats, 5-mm-diameter critical bone defects were created at the angle of the mandible. In the control group, the defect was not filled. The defects were grafted only Xenograft in Group 1, with Xenograft and tideglusib in Group 2, and with only autogenous bone graft in Group3, and with autogenous bone graft mixed with tideglusib in Group 4.

Results: Sterological analyses revealed that enhanced new bone formation in the Group 4 compare to Control and Group 1. Immunohistochemically marked expressions of BMP-2 and VEGF were observed in Group 4.

Conclusions: Our results demonstrated that Tideglusib, in combination with bone grafting has an adjuvant effect on BMP-2 and VEGF-A expressions that may accelerate bone regeneration.

Keywords: Autogenous Bone Graft, BMP-2, Bone healing, Tideglusib, VEGF-A.

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INTRODUCTION

A bony defect is defined as an acquired deformity from trauma, surgery, or congenitally. Different materials, including autogenous, xenogenous, allogenic, and alloplastic bone grafts, are available for the management of bony defects.[1] However, none of these materials are perfect because of harvesting problems, preparation difficulties, antigenicity, and consumption.[2] Therefore, researchers have focused on alternative biomaterials and applications, especially with the goal of accelerating bone formation and reducing healing time. Drugs or recombinant products have been combined with bone grafts, especially, to perform osteoinductive tasks such as growth factor expression.

Bone morphogenetic proteins (BMPs) are important factors in bone formation and regeneration Among the BMPs, BMP-2 has strong osteoinductive activity.[3] Regardless of the material, the development of a supporting vasculature concomitant with graft maturation is well documented and is a key determinant in optimizing bone regeneration at grafted sites. Vascular endothelial growth factor (VEGF) is a critical regulatory factor new vascular formation which is the most researched factor that has the strongest association with angiogenesis.

Tideglusib is a glycogen synthase kinase 3 (GSK-3) inhibitor used to treat neurological diseases such as Alzheimer's disease[4] and it has been reported to have a positive impact on the formation of dentin.[5]

Embryologically, tooth and bone are mineralized tissues derived from the neural crest with similar organic and inorganic matrices.[6] The organic matrix of dentin is composed of type I collagen (90%) similar to that of bone. The remaining 10% of the dentin matrix consists of biopolymers, lactate, citrate, lipid, osteocalcin, osteonectin, sialoprotein, phosphoprotein, which play a role in bone calcification and growth factors, including bone morphogenetic protein and insulin-like growth factor.

This study aimed to evaluate tideglusib mixed with bone grafts effect on formation and to evaluate the expression of Bone Morphogenetic Protein-2 and Vascular Endothelial Growth Factor by using immunohistochemical analysis.

MATERIAL AND METHOD

The study was approved by the Institutional Animal Care-Use Committee of Pamukkale University (protocol No PAUHADYEK- 2018/05). All experimental procedures on the animals complied with the Guidelines of Pamukkale University Care and Use of Laboratory Animals.

Study Design

Our study consists of five groups: one control and four experimental. Bony defects of 5 mm in diameter were created in the angle of the mandibles of 40 Wistar albino rats (250-300 g). The study groups were as follows: Control group: defects not filled, Group 1: xenograft material applied to the defect area, Group 2: xenograft and Tideglusib applied to the defect area, Group 3: autogenous graft material applied to the defect area, Group 4: autogenous graft and Tideglusib applied to the defect area (Figure 1).

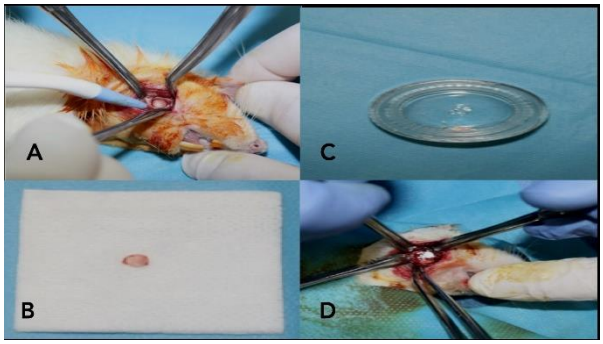

Figure 1.
A. Five-mm diameter bone defect made in angle of mandible.
B. Autogenous bone graft taken from rat's mandible.
C. Ideal sized autogenous graft material for grafting procedure.
D. Autogenous graft and Tideglusib application to defect area.

Tideglusib Preparation

Before the surgery, 50 nM Tideglusib (SIGMA AQ) was dissolved and diluted in dimethyl sulfoxide (DMSO) and mixed with the graft material.

Autogenous Graft Preparation

Autogenous bone graft material was taken from each rat's mandibular defect. The material was put into the sterile chamber of a grinding machine (KometaBio Dentin Grinder; Tidal Tech Dental, Turkey) that crushed and sterilized the material and

prepared particles (mean 0.75 mm in size; range 0.3-1.2 mm) suitable for the grafting procedure.

Surgical Procedures

The animals were anesthetized intraperiteonally with 1% ketamine–(Ketalar; Eczacibasi-Warner Lambert, Istanbul, Turkey) and 2% xylazine (Rompun 2%; Bayer, Istanbul, Turkey). The mandible was shaved and then scrubbed with sterile gauze soaked with an iodine solution. With sterile instruments and an aseptic technique, a 4- to 5-cm-horizontal incision was made in the right angle of the mandible. The subcutaneous tissue, muscles, and periosteum were dissected and reflected to expose the angle of the mandible. Only one bone defect (5 mm in diameter) was made in the mandibular bone of each rat with a trephine drill under constant irrigation with a 0.9% saline solution. No grafting material was used in the control group. Autologous mandibular onlay grafts were harvested from the right mandibular region, which was ground and used in Groups 3 and 4. Xenograft material (Bonefill, Bionnovation, Brasil) was used in Groups 1 and 2. The soft tissues were firmly repositioned and sutured (Resorbable 4.0 polyglycolic acid, Pegalak; Dogsan, Istanbul, Turkey). After the surgery, each rat was medicated with an intramuscular injection of Ceftriaxone, 50 mg/kg Rocephin; Deva, Istanbul, Turkey) and Carprofen, 4 mg/kg (Rimadyl; Pfizer, New York, NY) once daily for three days. The animals were euthanized 28 days after the surgery. The bones were dissected, and any signs of abnormal healing were documented.

Histopathological and Sterological Analysis

Specimens of the mandible with the bony defect were fixed in a 10% buffered formalin solution, after decalcification with a ready to use decalcification solution (Osteofast 1, Biognost, Zagreb, Croatia) for two weeks. Then the defective area was trimmed and tissue samples were washed under tap water during the 8 hours. After the routine tissue processing using an automatic tissue processor (Leica ASP300S, Leica Microsystems, Wetzlar, Germany), the specimens were embedded in paraffin. Then paraffin blocks were cut 5-µm thickness with a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany), Two different serial sections taken from each defective area and, stained with hematoxylin and eosin (HE).

Sections were coverslip and examined under a light microscope. Histomorphometric parameters were calculated for the total augmented area (TIA; mm^2), and the residual material area (RMA; mm^2). Osteoblasts and osteoclasts were counted in a 1.23 mm^2 area at ×400 magnification. Whole defective areas were examined at two dimensionally. All calculations were made 5 different areas of each sections of the defective areas.

For the stereological estimation of volume of newly formed bone area (VNFB; mm^3) light microscopic images via using point counting test grids were used. The point density of the point counting grids was designed to obtain an appropriate coefficient of error for interesting area in images of the serial sections in the 1mm thickness. Coefficient of error and coefficient of variation were estimated according to Gundersen and Jensen' formula. The test grid with systematic array of points was randomly placed on the screen of PC. For to estimation of the volume of each interesting area in all sections was used with following formula:

$$\text{Volume} = t \times a/p \times \sum p$$

('t', section thickness; 'a/p', representing area of each point on the point counting grid; '$\sum p$', (total number of the points hitting the interesting area) (Gundersen and Jensen, 1987) Mean values of each group results statistically examined. For histomorphometric and sterological analysis the Database Manual CellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan) was used. Histopathological changes were evaluated in a blinded manner by a specialized pathologist from another university who was unaware of the study design.

Immunohistochemical Method

For the immunohistochemical examination, the specimens were stained with BMP-2 (Anti-BMP2 antibody bs-1012R, 1/100 dilution) and VEGF-A (Anti-VEGF-A antibody bs-1957R, 1/100 dilution), by streptavidin–biotin complex peroxidase technique. Primary antibodies were purchased from Bioss (AQ USA). The sections were incubated with the primary antibodies for a period of 60 minutes, and

the immunohistochemistry was evaluated using a biotinylated secondary antibody and a streptavidin–alkaline phosphatase conjugate. As a secondary antibody, Expose Mouse and rabbit specific HRP/DAB Detection IHC Kit (AQ ab80436), and DAB (3,3-diaminobenzidine) (Abcam, UK) was used as a chromogen. The phosphate-buffered saline (PBS; pH: 7,2) solution was used for negative controls instead of primary antiserum. The evaluated by a specialized pathologist by manner. The immunohistochemical expressions were semiquantitatively scored from 0 to 3 (0=negative, 1=slight expression, 2=medium expression, 3=severe expression). After the microscopic examination (Olympus CX41 AQ), the Database Manual CellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan) was used for microtomographic and morphometric evaluation. Scores were statistically analyzed and the differences between the groups were determined.

Statistical Analysis

Statistical analysis of the immunohistochemical scores was calculated with the one-way ANOVA test by using the SPSS 15.00 statistical program. Significant differences between groups were detected with the DUNCAN test (α=0.005).

RESULTS

Histopathological Findings

The histopathological examination of the mandibular specimens revealed that the defect area was usually filled with connective tissue in all groups. However, in some groups, cartilage and bone were observed. Especially in the Control group, the center of the defect was empty. Maximum ossification was observed for the groups 3 and 4. Minimum ossification was observed in the Control group (Figure 2).

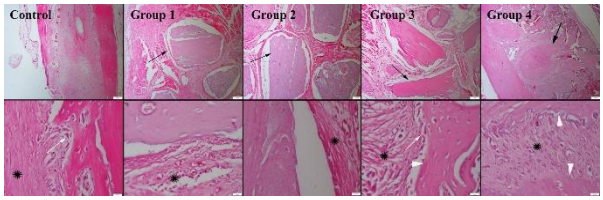

Figure 2.
Histopathological appearance of the bone defect area between the groups. (Upper row) graft materials (thin arrows) and new bone formation (thick arrow), HE, Bars= 200µm. (Below row) higher magnification of the fibrous tissue formations (stars), newly bone formations (white arrow heads) and osteoclasts (white arrows), HE, Bars= 20µm.

Sterological analyses showed that the total augmented area of the defects was significantly different. The marked amelioration was observed in Group 4. The new bone area was also higher in this group than in the Control and Group 1. The defect closure rate was also significantly higher in Group 4. The sterological findings and immunohistochemical expressions of BMP-2 and VEGF-A are shown in (Figure 3-5).

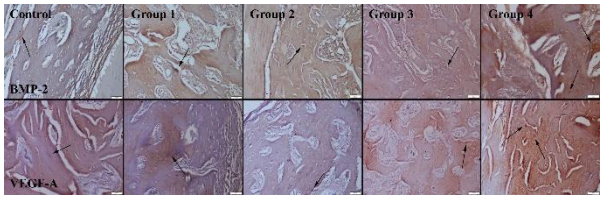

Figure 3.
BMP-2 and VEGF-A immunoexpressions (arrows) between the groups, the most marked increase in Group 4, streptavidin biotin peroxidase method, Bars= 20µm.

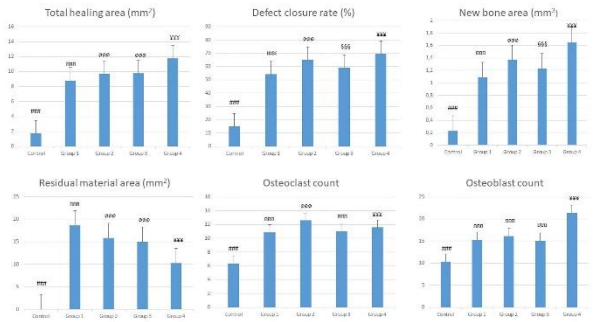

Figure 4
Statistical analysis results of the bone healing markers between the groups. Groups with superscripts of same letter are similar, different ones are statistically significantly different compared the control group (P<0.001).

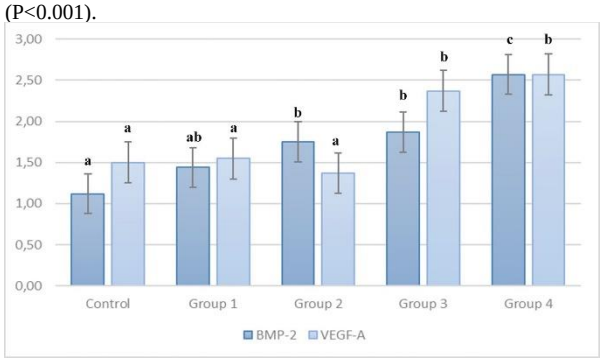

Figure 5
Graph of the statistical analysis results of BMP-2 and VEGF- A immunohistochemical scores. * One-way Anova Duncan test was used in statistical analysis.
** Groups with superscripts of same letter are similar, different ones are statistically significantly different for each marker (P<0.001).

DISCUSSION

If a graft has osteoinductive and osteoconductive effects, the healing rate and regeneration is improved compared with other materials.[7] Recently researchers have focused on enhancing the osteoconduction and osteoinduction capacity

with improved preparation design or by adding drugs that express growth factors.[8] Growth factors are chemical signals which regulate pathways involved in bone regeneration[8], which, at any given time, may not be observed under physiologic conditions. Growth factors effects are associated with significant changes in their local concentration and do not depend only on their presence or absence.[9] In our study, Tideglusib was combined with grafts to increase the local concentration of growth factors, especially BMP-2 and VEGF.

Combining bone grafts with other materials commonly focuses on enhancing regeneration via osteoinductive and vascularization processes. Studies have demonstrated that some materials that promote bone formation are LLP (lipid-lowering agent)[10,11], antioxidant molecules[12,13] or recombinant BMP-2.[14,15]

Growth factors bind to specific receptors and activate intracellular events by specific biochemical pathways and increase the chemotaxis of the same cellular elements in the regeneration area. Growth factors act on target cells by using specific receptors via specific biochemical pathways and also participate in the chemotaxis of the same cellular elements. The pathways have been reported in the literature. The Wnt pathway[16,17], the TGF-β/BMP superfamily[18], notch signaling[19], hedgehog proteins[20] and fibroblast growth factors (FGFs)[21] have all been identified in the molecular signaling of osteogenesis. Although the exact mechanisms of osteogenesis are complex and only partially elucidated, advances have been made regarding the initiation and molecular control of this process. The Wnt/β-catenin pathway organizes the postnatal bone acquisition by checking the differentiation of both osteoblasts and osteoclasts.[22] Wnt/β-catenin signaling is also essential for skeletal formation and development in the fetus and is known to be responsible for both osteoblast and chondrocyte differentiation.[23] This pathway not only plays a critical role in growth and development but in the maintenance of the mature skeleton.[24] Tideglusib is a non-competitive irreversible inhibitor of GSK-3ß and a member of the thiazolidinediodine family.[25] This drug activates the WNT/ß-catenin signaling pathway by inhibiting GSK-3ß.[26]

Our results demonstrated that Tideglusib increased BMP-2 expression. As Tideglusib has a positive effect on dentin regeneration by the Wnt pathway, we concluded that the same effect was seen in our study and may have been by the same pathway.

Chen *et al.*[27] reported in an in vivo study that BMP-2 induced the Wnt/ß-catenin pathway during ectopic endochondral ossification, chondrogenesis, and osteogenesis. Yuan *et al.*[28] reported that synchronous stimulation of BMP and Wnt/ß-catenin pathways indicated a scheduled function of BMP and Wnt signaling in coordination early tooth formation. Another study reported that suitable interference between BMP and Wnt signaling pathways was necessary for tooth formation.[29] According to these studies BMP-2 and WNT signaling pathways works in sync, and this condition is consistent with our results.

Therapeutic molecules reported to have a positive effect on BMP expression for enhanced bone healing include lithium chloride, sclerostin antibodies, strontium ranelate, LLP, and AZD2858. The effect of these drugs may be via the same pathway or others. Recently, Galli *et al.*[30] reported that activation of the canonical WNT/ß catenin pathway via lithium chloride increased osteoblast differentiation on hydrophilic modSLA surfaces. Also, Arioka *et al.*[26] reported that local application of lithium chloride and other GSK-3 inhibitors may expedite bone healing by activating osteoblastogenesis and suppressing osteoclastogenesis. LiCl increases implant osseointegration, implant fixation, and bone formation in osteoporotic conditions, so LiCl may be a promising curative material for avoiding implant failure and bone loss in patients with osteoporosis.[31]

Local drug application has the advantages of decreasing adverse systemic effects and systemic toxicity. Our study demonstrated that bone has a high tolerance for Tideglusib, and the dose and concentration were selected with the guidance of Neves *et al.*[5]

Vascularization is a critical factor for bone regeneration. Our results demonstrated that Tideglusib enhanced VEGF-A expression, which is associated with bone regeneration. In addition, this condition stimulated new vessel production and circulation and increased osteogenic cell formation, increasing regenerative capacity. Some drugs like LLP[32] and Link si-RNA[33] are both VEGF and BMP-2 releasing. Similarly, our results showed that Tideglusib promotes the release of both BMP-2 and VEGF. If used together with BMP-2, VEGF, and bFGF, it might synergistically support osteogenic differentiation, with low concentrations. BMP-2, and VEGF-A influence each other during bone regeneration; therefore, both BMP-2 and VEGF-A release is anticipated to increase bone healing compared with each alone.[34,35,36] Similarly, in our study, the synergistic effect of BMP-2 and VEGF might increase bone regeneration. Tideglusib is a drug used for Alzheimer therapy that has been shown to contribute to dentin regeneration.[5]

CONCLUSIONS

These study findings revealed that this drug effective for new bone and vessel formations that support to healing. BMP-2 and VEGF have pivotal role for bone and tissue remodelling. Our results demonstrated that this drug has a positive impact on bone regeneration owing to the similarity of bone and dentin. Additional studies are necessary to determine the mechanism of this drug.

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DECLARATIONS OF INTEREST

"No potential conflict of interest was reported by the authors."

ÖZ

Bu çalışmanın amacı, otojen ve ksenojen kemik greftlerle karıştılarılan Tideglusib'in ratlarda oluşturulan kemik defektlerindeki rejeneratif potansiyelinin incelenmesidir. Çalışma bir kontrol ve dört deney grubu olmak üzere beş gruptan oluşmaktadır. Rat mandibula angulus bölgesinde, 5 mm çapında kritik kemik defekti oluşturulmuştur. Kontrol grubunda defektler boş bırakılmıştır. Grup 1 de defektlere sadece Ksenojen kemik greft, Grup 2 de Ksenojen kemik greft ve Tideglusib, Grup 3 de sadece otojen kemik greft ve Grup 4 de ise otojen kemik greft tideglusib ile karıştırılarak uyugulanmıştır. Sterelojik analiz sonuçlarına göre yeni kemik formasyonu, Grup 1 ve Kontrol Grubu ile karşılaştırıldığında Grup 4' de daha fazla oluşmuştur. Grup 4 de immunohistokimyasal olarak daha fazla hacimde BMP-2 ve VEGF ekspresyonu gözlemlenmiştir. Çalışmamızın sonuçları; Tideglusib ile karıştılan kemik greftlerin BMP-2 ve VEGF ekspresyonununu artırdığını ve bu durumunda kemik rejenerasyonunu geliştirdiğini göstermektedir.

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