Cumhuriyet Dental Journal 2017; 20(2):
Doi: 10.7126/cumudj.345934

EFFECT OF LOCAL RIFAMYCIN APPLICATION ON EXPRESSION OF BMP-2 AND BONE REGENERATION

Yerel Rifamicin Uygulamasının Bmp-2 ve Kemik Rejenerasyon Açısından Etkisi

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Makale Kodu/Article Code *: 189624*
Makale Gönderilme Tarihi *: 17.05.2016*
Kabul Tarihi *: 27.05.2016*

ABSTRACT

Objectives: The aim of this study was to evaluate effect of local rifamycin application on BMP-2 expression and bone healing.

Materials and Methods: A standardized 5.0-mm- diameter critical size bone defect was created mandible angulus region. In the control group (8 rats) defects were left empty. In the Group 1 (n=8 rats) defect was irrigated with rifamycin solution and 25 mg rifamycin solution injected defect area at 1, 3, 7 days after surgery. In the group 2 (n=8 rats) defects were grafted with a gelatin sponge mixed 25 mg rifamycin solution. Rats were sacrificed at 21 days after surgery. Histological slides were prepared from defect site for both immunohistochemical analysis (bone morphogenetic protein-2 (BMP-2) antibody) and histomorphometric analysis. Data were analyzed using Mann Whitney U and Kruskall Wallis test.

Results: The average new bone formation, number of osteoblast and new vessel formation count were increased more in both of experimental groups in comparison with control group. Anti-BMP-2 labelling (Cell count) was increased more in both of experimental groups in comparison with control group.

Conclusion: Local rifamycin application has positive effects on BMP-2 expression and bone regeneration at critical sized bone defects.

Keywords: Rifamycin, critical sized bone defect, bone regeneration, bone morphogenetic protein – 2

ÖZ

Amaç: Bu çalışmanın amacı lokal rifamisin uygulamasının kemik iyileşmesi sırasında BMP–2 salınımı üzerine etkisinin değerlendirilmesidir.

Materyal ve method: Rat mandibula angulus bölgesinde standart olarak 5 mm çapında kritik boyutta kemik defektleri oluşturulmuştur. Kontrol grubunda (8 rat) defektlere herhangi bir uygulama yapılmamıştır. Birinci deney grubunda (8 rat) defekt bölgesi rifamisin solüsyonu ile irrige edildikten sonra, defekt bölgesine 1, 3 ve 7. günlerde 25 mg rifamisin solüsyonu enjekte edilmiştir. İkinci deney grubunda (8 rat) defekt bölgesi 25 mg rifamisin solüsyonu ile karıştırılmış gelatin sponge ile greftlenmiştir. Cerrahiden 21 gün sonra ratlar sakrifiye edilmiştir. Defekt bölgesinden hem immünhistokimyasal analiz (kemik morfogenetik protein –2 antibody) için hem de histomorfometrik analiz için histolojik kesitler hazırlanmıştır. Elde edilen verilerin analizi Mann Whitney U ve Kruskall Wallis testi kullanılarak yapılmıştır.

Bulgular: Deney grubunda kontrol grubuna göre ortalama yeni kemik formasyonu, osteoblast sayısı ve yeni damar oluşum sayısında artış olduğu görülmüştür. Her iki deney grubunda da anti–bmp–2 ile işaretlenmenin (hücre sayma) kontrol grubuna göre daha fazla olduğu görülmüştür.

Sonuç: Kritik boyutta kemik defektlerine lokal olarak rifamisin uygulamasının BMP–2 salınımı üzerine pozitif etkileri olduğu tespit edilmiştir.

Anahtar Kelimeler: Rifamisin, kritik boyutta kemik defekti, kemik rejenerasyonu, kemik morfogenetik protein – 2

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INTRODUCTION

Despite improvements in antibiotic therapy and surgical procedures, bone defect reconstruction, bone infection, and bone graft resorption, are still remaining as problems in oral and maxillofacial surgery. Bone reconstruction success depends on the size of defect, regeneration capability, stability, vascularization, and infection.

Infection is a crucial factor and can have hazardous effects on bone healing.[1,2] Bone resorption increases at lower pH levels, resulting in bone augmentation failures.[1,2] Infection inhibits cytokine release, resulting in compromised bone wound healing or bone graft resorption.[3] Local antibiotics are commonly used to treat bone infections such as osteomyelitis, or perimplantitis, to prevent initial infection risk, or, recently, as prophylactic treatment added to bone grafting materials empirically.[4] Bone graft vascularization and blood supply is poor; therefore, systemic use of antibiotics cannot reach adequate levels of antibacterial concentration. This dilemma can be resolved by local delivery of antibiotics. Locally administrated antibiotics may reach a twenty-fold higher concentration in graft site versus intravenous administration.[4]

The infected bone area is must provide a framework of both osteoinductive and osteoconductive materials, along with antibiotics.[5] An osteoconductive carrier system delivering antibiotics and osteoinductive agents locally would be an ideal and novel approach for treating infected bone defects.[6] Several osteoconductive bone substitutes and natural polymers are used as local antibiotic delivery vehicles. Collagen is widely used as a carrier material for drug delivery and provides a physical scaffold around the antibiotic, mechanically limiting fluid flow, or as a scaffold for bone engineering. Collagen can also stimulate the proliferation of osteoblasts and the production of collagenous callus tissue, thereby aiding the formation of new bone.[7]

The healing of bone defects involves in three mechanisms; osteogenesis, osteoconduction and osteoinduction.[8,9] Osteoinduction provide the biological stimulus along with signal pathways for the transformation and stimulation of stem cells into bone-producing cells during bone regeneration.[9,10] Bone morphogenetic Proteins (BMP) are members of the transforming growth-factor superfamily β that are known to regulate the differentiation and proliferation of several cells.[11] BMP-2 has the highest osteoinductive capacıty among BMPs.[12] Release of BMP-2 begins in the early stages of the bone healing process; recombinant BMP-2 is used to induce bone formation in reconstructive procedures.[11,13]

Rifamycins are semisynthetic bactericidal antibiotics, and that are effective against Gram positive and Gram negative bacteria. Rare allergic reactions and a few adverse effects may occur after local application of rifamycin.[14,15] Rifamycins are used for surgical site infection in orthopedic and maxillofacial surgeries and are well tolerated by bone tissue. Previous studies reported rifamycin may positively affect bone tissue, extraction socket healing, and osteomyelitis treatment.[16-19] In the literature, there is a little knowledge about the effect of antibiotics on BMP expression. Previously Ufuk *et al.*[20] reported rifamycin is a suitable solution for bone decontamination and may induce BMP-2 expression. We aimed to investigate the effect of local rifamycin application on BMP-2 expression and bone formation.

MATERIAL AND METHODS

All animal procedures were approved by the Institutional Animal Care ε Use Ethical Committee of Cumhuriyet University (permit no: 2011-248), and their care was in accordance with institution guidelines. Wistar albino rats (n=24) were used for this study. The rats were at

the adult stage and weighed approximately 300 g. The animals were kept in cages and fed a solid diet and water and libitum.

The 24 rats were divided into one control group (n = 8) and two experimental groups of 8 (group 1 and group 2). Standardized 5 mm diameter critical-size bone defects (CSDs) were created in the right mandible angulus. CSDs were left empty in control group. Defects in experimental group1 were irrigated with Rifamycin SV (Rifetem 250 mg, Ulagay, İstanbul, Turkey; Figure 2) and 25 mg Rifamycin SV was injected with an insulin injector at the defect area on the first, third, and seventh days after surgery. Defects in experimental group 2 were grafted with a gelatin sponge (Spongostan, hemostatic absorbable gelatin sponge, Ferrosan, Denmark) mixed with 25 mg Rifamycin Solution (Figure 1).

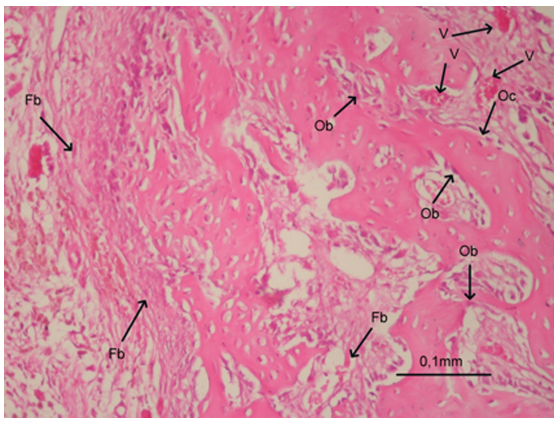

Figure 2. Histologic evaluation of defect area (Ob:osteoblast, Oc: osteoclast, Fb: fibroblast, V: vessel). Bar: 100µm. Haematoxylin-and-eosing staining.

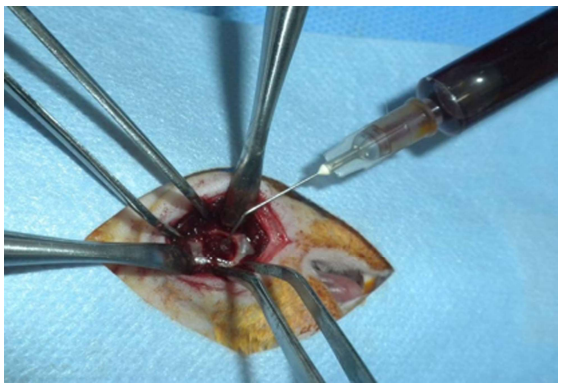

Figure 1. Irrigation of rifamycin solution.

Surgical Procedure

For all surgical operations, the rats were anesthetized with an intraperitoneal injection of 3 mg/kg Xylazine (Rompun 2%; Bayer, İstanbul, Turkey) and 90 mg/kg Ketamine HCl (Ketalar; Eczacıbaşı- Warner Lambert, İstanbul, Turkey). Defects were created on right the mandible angulus.

The skin of the mandible was shaved and disinfected with iodine. An incision was made inferior to the angle of the mandible extending to the mandibular bone and the periosteum of the mandible was ablated. A standardized 5 mm diameter defect was created using a surgical trephine with an internal diameter of 5 mm. Subcutaneous tissues were sutured with 5-0 Vicryl (Pegelak, poly-glycolide-co-lactide) [PGLA]; Doğsan, Trabzon, Turkey), while the skin flaps were closed using 5-0 nylon sutures (Ethicon, Edinburgh, UK) and allowed to heal by primary intent. All the animals received a subcutaneous antibiotic and analgesics: 25mg/ kg ceftraixone (Rocephine, Roche, Basel, Switzerland) and 4 mg/kg carprofen (Rimadyl, Pfizer, New York, NY, USA), respectively, for 3 days at every 24 hours, starting immediately after operation.

Histologic and Immunohistochemical Analysis

The rats were sacrificed on the 21st day after surgery with an overdose of sodium pentobarbital. The mandible bones were excised and separated into hemimandibles together with the surrounding tissue, and fixed in 10% buffered paraformaldehyde for 48 hours; they were then decalcified in ethylenediamine tetra-acetic acid (EDTA) solution. The tissue specimens were prepared in an autotechnicon, embedded in paraffin, and sectioned (5 µm) with a microtome. The sections were stained with haematoxylin-eosin. The tissue sections were examined and imaged by means of a Nikon Eclipse E400 light microscope and Nikon Coolpix 5000

digital camera. All photographs were then transferred into a PC environment and analyzed (Clemex Vision Lite 3.5 Image Analysis, Clemex Technologies, Longueuil, Quebec, Canada). The length was calibrated by comparing the photograph of the specimen with the photograph of the Nikon micrometer microscope slide, which was taken under the same magnification. An area of 0.4 mm^2 was designated using the Clemex Vision Lite 3.5 Image Analysis program, and osteoblasts, osteoclasts, and new bone areas were marked with the same Image Analysis program in a 0.4 mm^2 area. Damaged cells were not evaluated. The marked cells were counted automatically with the same image analysis program. The histological procedure was performed in a different department, pathologists were blinded to the animal group's information, and measurements were evaluated with the image analysis programme for reproducibility of procedure.

For immunohistochemical staining, the sections (5 µm) were stained with hematoxylin and eosin and monoclonal antibodies for analysis of BMP-2 expression (rhPro-BMP-2, clone: 253717, mouse monoclonal antibody, [R&D Systems, Inc]). Immunostained cells were evaluated in the same manner described above. First, a 0.4 mm^2 area was designated using the image analysis program; then positive-stained cells were then marked with the same image analysis program in a 0.4-mm^2 area. Damaged cells were not evaluated. The marked cells were counted automatically with the same image analysis program. The measurements were repeated 5 times, and then the average data were obtained.

The mean (SD) was calculated for each group. The data were analyzed using Kruskal Wallis analysis and Mann-Whitney U- test. Probabilities of less than .05 were accepted as significant.

Histometric Results

The histological specimens of all groups are shown in Figure 2-5.

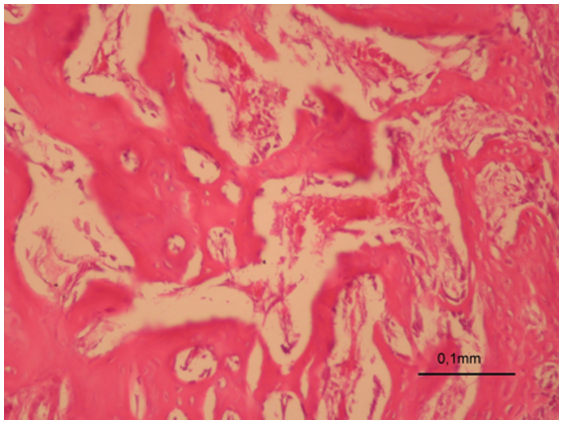

Figure 3. Histological evaluation of Control Group. Bar: 100µm. Haematoxylin-and-eosing staining.

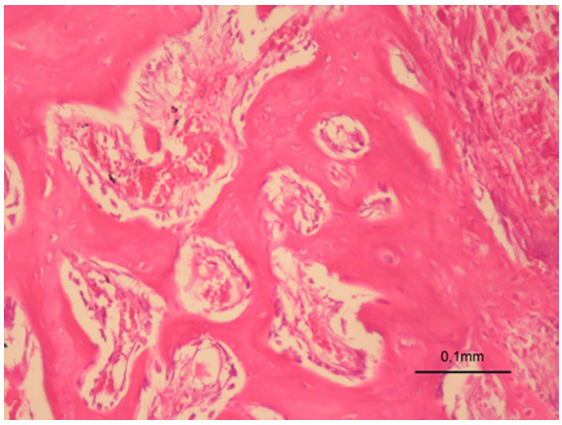

Figure 4. Histological evaluation of Group 1 (Rif injected). Bar: 100µm. Haematoxylin-and-eosing staining.

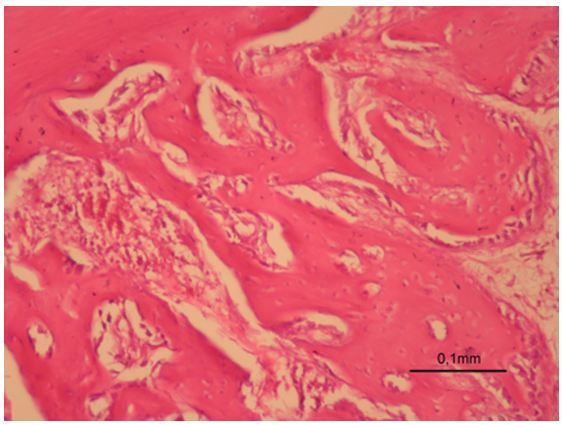

Figure 5. Histological evaluation of Group 2 (Rif mixed with gelatin sponge). Bar: 100µm. Haematoxylin-and-eosing staining.

New bone area, and osteoblast, osteoclast, fibroblast and new vessel counts were evaluated. New bone volume, as well as osteoblast, fibroblast and new vessel counts were higher for group1 and group 2 than the control Group ($p<0,05$). No statistically significant differences were found in terms of new bone area or osteoclast, fibroblast, and new vessel counts between groups 1 and 2 (Table 1).

Table 1. Mean (SD) histometric results of defect regions of selected 0.4 mm^2 area.

Variable	Control group Mean (SD)	Group 1 (R.I) Mean (SD)	Group 2 (G.S) Mean (SD)	P- value
New bone area (mm^2)	78583,71 (4834,92)	88789,09 (3643,30)	89287,44 (3413,64)	KW: 12,62 P: 0,001*
Osteoblast Count	15,20 (2,69)	17,30 (1,63)	19,40 (2,06)	KW: 4,75 P: 0,029 *
Osteoclast Count	1,10 (1,37)	1,10 (0,56)	0,70 (0,67)	KW: 0,69 P: 0,403
New Vessel Count	2,80 (0,78)	4,10 (0,73)	4,20 (0,78)	KW: 8,49 P: 0,004 *
Fibroblast Count	19,60 (2,67)	22,40 (2,87)	21,80 (2,65)	KW: 4,52 P: 0,033*

Data were analysed using Kruskall Wallis and Mann Witney U test. The level of significance was set at $P < 0.05$. * : $P < 0.05$. R.I: Rif injection and irrigation. G.S: Rif mixture with gelatin sponge.

Immunohistochemical Results

The immunohistochemical specimens of all groups are shown in figures 6-7. The BMP-2 counts in group 1 and group 2 were more statistically significantly higher when compared to the control Group ($p<0,05$). No statistically significant differences were seen in terms of the BMP-2 count between group 1 and group 2 (Table 2).

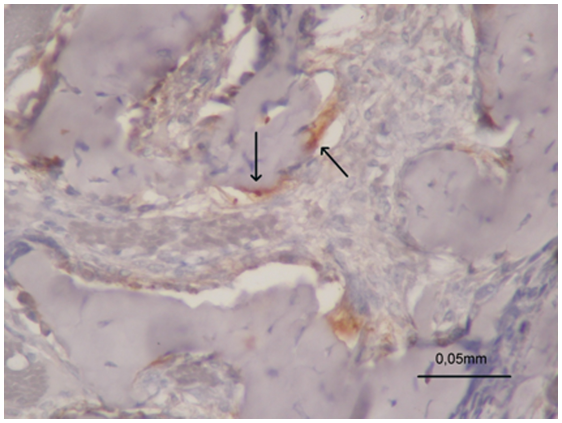

Figure 6. Immunohistochemical analysis of Control group defect area. The sections were stained with monoclonal anti-human Pro-BMP-2 antibody. Bar: 50µm.

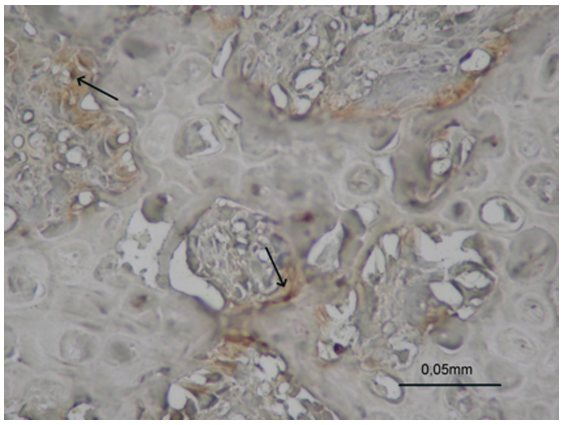

Figure 7. Immunohistochemical analysis of Experimental Group defect area. The sections were stained with monoclonal anti-human Pro-BMP-2 antibody. Bar: 50µm.

Table 2. Mean (SD) immunhistochemical measurements of defect regions of selected 0.4 mm^2 area.

Variable	Control group Mean (SD)	Group 1 (R.I) Mean (SD)	Group 2 (G.S) Mean (SD)	P- value
Anti-BMP-2 labelling (cell count)	4,00 (0,94)	6,40 (0,96)	6,40 (0,96)	KW: 12,27 P: 0,001*

Data were analysed using Kruskall Wallis and Mann Witney U test. The level of significance was set at $P < 0.05$. *: $P < 0.05$. R.I: Rif injection and irrigation. G.S: Rif mixture with gelatin sponge.

DISCUSSION

Local antibiotics were preferred for reducing risk of initial surgical infection, adverse systemic effects, systemic toxicity, and unnecessary high dose of antibiotic intake.[21] However dose-dependent and systemic administered rifamycin's possible cytotoxic effects on the cells were demonstrated in vitro studies.[22,23] On the other hand; previous studies and our study demonstrate higher osteoblast counts, enhanced bone formation[19], high tolerance by bone[17,18], and no histological damage.[16] Cytotoxicity may associated with dose, concentration, the type of antimicrobial and exposure time.[2] The selection of appropriate local antibiotics should consider antimicrobial effects, cytotoxicity, concentration and dosage factors. In our study the dose and concentration of rifamycin were selected with the guidance of Ferhan *et al.*[16], and Sivollella *et al*.[18] The antimicrobial effect of rifamycin was demonstrated by the same studies.[16,18]

There is little information available regarding the relationship between antibiotic

delivery and tissue regeneration. Recently, local delivery growth factors or antibiotics delivered from an implanted biomaterial have been used as novel approaches to stimulate bone regeneration areas of infected bone or compromised bone healing. Our methods present to overcome this situation basically and at lower cost.

In addition to rifamycins antibacterial use, their other anti-inflammatory effects have been shown in previous studies. Rifamycins are used for the treatment of rheumatoid arthritis[24] or chronic arthritis by direct intra-articular injection.[25] Anti-inflammatory and immunomodulatory effects have been shown to inhibit cytokine and chemokine synthesis by Rosetta *et al*.[24] The advantages of our study are positive effects on bone formation and, stimulating effects on BMP-2 release with other beneficial properties of rifamycin. Doxicyclin, gentamycin, and rifamycin have shown enhanced bone formation.[19,26,27] On the other hand controversial study reported antibiotics can inhibit bone formation.[28] Negative results may be related to dosage and concentrations.

New vessel formation was enhanced in our rifamycin groups. BMPs can stimulate vascular endothelial growth factor (VEGF) expression and promote angiogenesis.[29,30] However a previous study reported there was no significant differentiation in VEGF expression between control and experimental groups.[20] Further study is required regarding rifamycin's effects on angiogenesis and VEGF expression.

We investigated rifamycin induced BMP-2 expression. The mechanism remains unclear. This effect may be a pleotropic effect the same as statins. We thought that increased bone formation was related to BMP-2 release. Muthukuru *et al.*[31] reported doxycyclin was a stronger inducer of alkaline phosphatase expression but combined with BMP-2, counteracted the ınduction of osteogenic mediators. Wübbenhorst *et al.*[32] investigated whether tetracycline had a positive effect on inducible BMP-2 expression. Liu *et al*.[33] reported doxycyclin induced Smad 1C expression and indirect effect on BMP's influence. Smads are a group of intracellular effectors of the pathway of BMP and expressed by BMP.[33] Unfortunately there is not enough information about the mechanisms of rifamycin's effect on BMP expression. Our study may be a pioneer study for the pleotropic effect of rifamycin on BMP expression.

Our results demonstrate higher osteoblast counts at the collagen delivery system. This result may be related to the osteoconductive properties of collagen. BMP-2 expression and new bone area were not different between group 1 and group 2. Carvalho *et al*.[17] used rifamycin for the treatment of fibrinolytic alveolitis and observed that only rifamycin irrigation had better bone formation than mixed rifamycin and gelfoam. Kaya *et al.*[19] reported rifampin mixed with allogenic bone grafts could have a negative effect on bone formation compared to rifamycin-only irrigation and mixed with other graft types. In our study bone defects were enclosed by surrounding tissues; however, in clinical applications, a collagen delivery system may be useful in open bone defects.

We conclude that the simple application and beneficial effects of rifamycin used for extraction socket preservation, sinus bone augmentations, bone infections, or mouthwash following third-molar surgery. This study may be a guiding light of the pleotrophic effect (BMP-2 expression) or pathway of rifamycin induced BMP-2 expression. We conclude that rifamycin is the best local antibiotic which clinicians add bone grafts safely.

Acknowledgements

This work was supported by the Scientific Research Project Fund of Cumhuriyet University under Project number DİŞ-108.

Ethical approval

İlker ÖZEÇ, Asistant Professor, DDS, Phd, Medical Ethics Committee of Medical Faculty, University of Cumhuriyet. Reference No: B. 248 05-05-2011.

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