Original Research Article

Effects of Systemic Administration of Resveratrol on New Bone Formation in Critical-Sized Bone Defects in Rats

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Abstract

Antioxidant agents stimulate new bone formation during the process of repair of bone defects. This study aimed to examine the effects of systemic administration of resveratrol, an antioxidant agent, on new bone formation in a rat model of experimentally induced bone defects. The study consisted of four groups. Each group was divided into 2 subgroups on the basis of the date on which the animals were sacrificed. We formed a 5-mm bone defect in the right mandible of the animals in each group. Control group (C) received no further treatment, first experimental group (G) received a bovine bone graft, second experimental group (R) received systemic administration of resveratrol (10 mg/kg), third experimental group (GR) received systemic administration of resveratrol (10 mg/kg) with a bovine bone graft. The results of histopathological examination on the 14th and 28th day showed significant differences in the degree of ossification, fibrosis, angiogenesis, and the density of inflammatory cells between the groups ($p < 0.05$). Results of biochemical analysis showed significant differences in the serum levels of bone morphogenetic protein-2 (BMP-2) and tumor necrosis factor α (TNF-α) between the groups ($p < 0.05$). Thus, our results showed that systemic resveratrol administration with graft placement may have positive effects on bone healing in critical-sized bone defects.
Keywords: Bone, graft, healing, rat, resveratrol.

INTRODUCTION

Bone tissue is the primary working area for oral and maxillofacial surgery. Regenerative properties of the bone tissue make it able to respond to any injury over the lifetime with repair process without scar formation. Small bone defects show spontaneous regeneration without any need for surgical intervention however defects over a certain size does not. For larger bone defects, surgical intervention is necessary [1].

Researchers have been studying on grafts and biomaterials that could stimulate new bone formation during the repair of bone defects. Experimental and clinical studies were carried out using graft materials to achieve faster, less problematic healing and to prevent the occurrence of adverse effects. While autogenous bone grafts are still considered to be the gold standard for treatment of defects that fail to heal spontaneously, some disadvantages led to use of other types of grafts (allograft, xenograft, alloplastic) and gave rise to research of their effectiveness when used in combination with other treatment modalities. Bovine graft is structurally very similar to human cancellous bone and has osteoconductive and biocompatible properties [2]. Thus, currently deproteinized bovine bone grafts are commonly used in a variety of surgical operations [3].

Recent studies have demonstrated the negative effects of free radicals on bone healing. Certain plants and various herbal extracts containing polyphenolic substances were found to show substantial antioxidant effect on free radicals [4].

Resveratrol (3,4,5-trihydroxystilbene) is a compound with antioxidant properties which is in natural phytoalexin structure. It is abundant in fruits including mainly grapes, peanuts, raspberries, mulberries, blackberries, plums and red wine and in the roots of a plant called *Polygonum cusbidatum.* Clinical and experimental studies have shown that resveratrol has anticarcinogenic properties and protective effects against cardiovascular disorders and it inhibits platelet

aggregation [5, 6]. Recent studies have also shown that, resveratrol has considerable effects on bone formation [7-9].

The aim of the present study is to evaluate the effects of resveratrol on the repair of bone defects when administered systemically and to enhance the quantity and quality of newly formed bone.

EXPERIMENTAL SECTION

Study Design

The study protocol was independently reviewed and approved by the Institutional Review Board of Cumhuriyet University and the Committee for Ethical Treatment of Experimental Animals (date and number, 12.12.2014-74).

The study was performed using male adult Wistar albino rats (n = 56) weighing approximately 250-300 g. The mean age of the animals was 12 weeks, and the rats were in good health as determined by a veterinary examination. The animals were kept in a 12-h light/12-h dark cycle with free access to food and water. The room temperature was set at 22 ± 2°C. The risk of contamination was eliminated by maintaining the relative humidity at 30-45% and supplying filtered air in the room where the rats were kept.

All experimental animals (56 rats) were divided into 4 main groups (C, G, R, and GR). Then, each group was divided into 2 subgroups on the basis of the day on which the animals were sacrificed, i.e., the 14th or 28th day (Table 1).

Surgical Technique and Resveratrol Application

The animals were anesthetized using an intramuscular injection of 3 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey) and 90 mg/kg ketamine HCL (Ketalar; Eczacıbaşı-Warner Lambert, Istanbul, Turkey).

The skin overlying the angle of the right mandible of each rat was shaved. The surgical site was prepared using povidone-iodine (Batticon standard solution, Adeka, Turkey) and covered with sterile drapes exposing the operation site. We performed a 1-cm incision on the mandibular angle 1 cm below the base of the mandible to remove the skin, subcutaneous tissue, and periosteum. The skin flap was elevated to expose the surface of the mandibular bone. A trephine drill was used to remove a critical-sized (5 mm in diameter) fragment of the bicortical bone under irrigation with saline solution (Fig-1). We created a 5-mm critical-sized defect in the mandibular angle area in the control (C) and resveratrol (R) groups with no further intervention on the defect area. In the graft (G) and graft + resveratrol (GR) groups, the critical-sized bone defects were filled with deproteinized bovine bone graft (Bio-Oss®; Geistlich Biomaterials, Wolhousen, Switzerland) (Fig. 2). After the surgery, the skin, muscle, and subcutaneous fascia were closed by suturing the skin flap in its original position using 5-0 polyglactin 910 (Vicryl, Johnson & Johnson/Ethicon) sutures. The experimental animals received an intramuscular injection of 4 mg/kg carprofen (Rimadyl, Pfizer) as an analgesic and 25 mg/kg ceftriaxone (Rocephin; Roche) as an antibacterial agent for 5 days during the postoperative period.

Resveratrol (Sigma, catalogue no: R5010, USA) was weighed to prepare a dose of 10 mg/kg/day for each rat. Resveratrol was dissolved in ethanol and then diluted with saline (1:3) and a fresh solution was prepared daily. Starting from the day of the operation, we administered 10 mg/kg/day resveratrol solution systemically to rats in the R and GR groups via oral gavage once a day.

The blood samples were obtained, and then, the animals were sacrificed on the 14th and 28th day using 200 mg/kg sodium pentobarbital (Pentothal; Abbott, USA). The experimental side of the rat mandibles were dissected and removed with the surrounding soft tissue and stored in 10% formalin solution for histopathologic examination. The blood samples collected were transferred into blood tubes and centrifuged (NF 1200R centrifuge) for examination using enzyme-linked immunosorbent assay (ELISA).

Histopathological Evaluation

Soft tissues were removed from the tissue samples that had been fixed in 10% formalin solution and the bone tissue was decalcified in 1% nitric acid solution. After decalcification, the tissues were examined using routine procedures and paraffin blocks were prepared. We prepared 5-µm-thick sections from these paraffin blocks and stained them with hematoxylin-eosin for histopathological examination. The sections were examined under a light microscope (Leica DM 2500), and bone healing was evaluated by examining the levels of ossification, angiogenesis, inflammatory cells, and fibrosis.

Biochemical Evaluation

A biochemical assay was performed according to the protocols described in the kit. We prepared serial dilutions using the standard solution to achieve concentrations of 62.5, 125, 250, 500, and 1000 pg/mL of bone morphogenetic protein-2 (BMP-2) and 15.6, 31.2, 62.5, 125, and 250 pg/mL of tumor necrosis factor α (TNF-α) and loaded them into the wells. Further, we added 100 µL sample diluent buffer, 100 µL sample, and 100 µL of the standard into the wells and incubated the plates at 37°C for 90 min. After removing the cover and removing the contents of the plates, we added 100 µL of biotinylated anti-rat TNF-α antibody into each well and incubated at 37°C for 60 min. Then, the plate was washed three times with 0.01 M phosphate-buffered saline (PBS) each for 1 min. Subsequently, we added 0.1 mL of reconstituted ABC solution into each

well, and the plate was incubated at 37°C for 30 min. The plates were washed five times and 90 µL of the prepared TMB color reagent solution was added to the plates, and the plates were incubated at 37°C for 25-30 min. Finally, we added 100 µL TMB stop solution into the wells and the reaction was terminated after development of yellow color. The absorbance was measured at 450 nm within 30 min of adding the stop solution by using an ELISA microplate reader (Thermo Multiskan GO Microplate Spectrophotometer).

STATISTICAL ANALYSIS

The study data were uploaded to the SPSS (Version 22.0) software and analyzed using Kruskal-Wallis test and Mann-Whitney U test when parametric test assumptions were not met and analysis of variance (ANOVA) and Tukey's test when parametric assumptions were met. The margin of error was set at 0.05.

RESULTS

Clinical findings

The rats tolerated the surgical operation well. The animals were in good health without any postoperative infection, and their feeding was not affected adversely throughout the study.

Histopathological Findings

The histological sections obtained from the groups were examined under the microscope to determine the levels of ossification, angiogenesis, inflammatory cells, and fibrosis (Fig 4-7).

We observed a significant difference in the scores of ossification, angiogenesis, inflammatory cells, and fibrosis between the groups ($p < 0.05$, Fig-3).

We observed a significant difference in the scores of ossification on the 14th and 28th days between the groups ($p < 0.05$). The GR group showed a significantly higher degree of ossification than the control group ($p < 0.05$, Fig-3).

The score of angiogenesis scores was significantly higher in the GR group than in the control group on the 14th and 28th days ($p < 0.05$, (Fig-3).

The score of fibrosis was significantly higher in the GR group than in the control group on 14th day ($p < 0.05$, Fig 3).

A significant difference was observed in the number of inflammatory cell between the C and GR groups on the 14th day ($p < 0.05$), and the number of inflammatory cells in the GR group were lower than those in the control group (Fig-3).

Biochemical Findings (ELISA Findings)

Bone healing was assessed biochemically by determining the levels of BMP-2 and TNF-α. The levels of BMP-2 on the 14th and 28th days were significantly different between the groups. The BMP-2 levels increased in the groups receiving resveratrol, and compared to the control group, the GR group showed a significant increase in the BMP-2 levels ($p < 0.05$, Table-2).

In addition, we observed a significant difference in the concentrations of TNF-α between the groups ($p < 0.05$). The levels of TNF-α on the 14th and 28th days were lower in the groups receiving resveratrol (R and GR) than in the other groups (C and G) (Table-3).

Table 1: Study groups

Groups	Group sacrificed after 14 days	Group sacrificed after 28 days	Number of rats per group
Control (C)	7	7	14
Graft (G)	7	7	14
Resveratrol (R)	7	7	14
Graft + Resveratrol (GR)	7	7	14
Total	28	28	56

Table-2: Comparison of the levels of bone morphogenetic protein-2 (BMP-2) between the groups

Groups	14. days		28. days	
	$\bar{X}\pm Ss$	Median	$\bar{X}\pm Ss$	Median
C	1,45±0,24	1.57	1,31±0,21	1.33
G	1,21±0,32	1.15	0,94±0,13	0.94
R	1,44±0,20	1.4	1,57±0,30	1.16
GR	2,26±0,42	2.06	1,20±0,14	1.16
Result	KW= 2,25		KW= 1,08	
	p= 0,0522*		p= 0,782	
$\bar{X}$: Mean value, Ss: Standard deviation, KW: Kruskal Wallis Test, *: ($p<0,05$)				

Table 3: Comparison of the levels of tumor necrosis factor α (TNF-α) between the groups

Groups	14. days	28. days
	$\bar{X}\pm Ss$	$\bar{X}\pm Ss$
C	82,27±3,67	65,00±2,43
G	75,88±5,29	66,51±4,07
R	69,70±6,07	57,48±4,55
GR	70,05±5,00	56,77±5,55
Result	F=9,48	F=9,54
	p=0,001*	p=0,001*

$\bar{X}$: Mean value, Ss: Standard deviation, F: Analysis of variance (ANOVA), *: (p<0,05)

Fig-1: A critical- sized bone defect formed following osteotomy

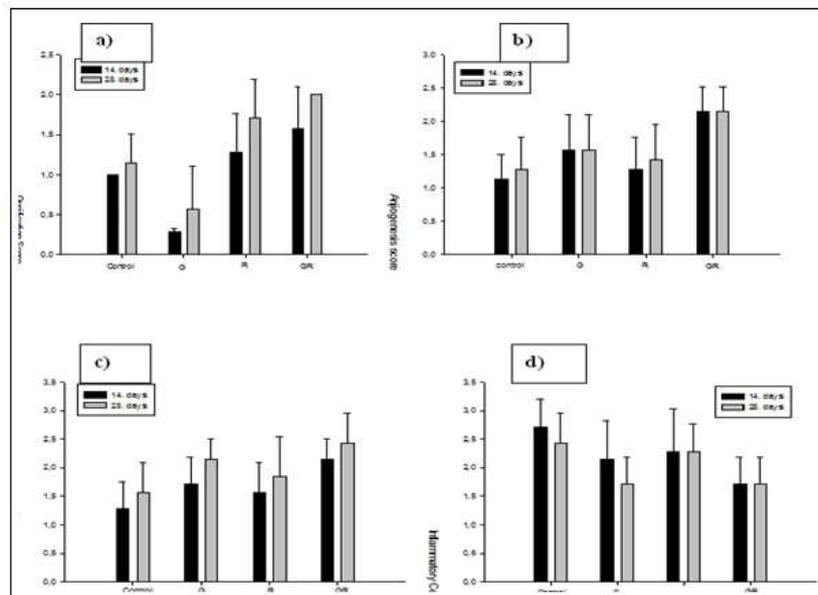**Fig-2: Graft material applied on a critical- sized bone defect**

Fig-3: Comparison of the amounts of ossification (a), angiogenesis (b), fibrosis (c), and inflammatory cells (d) in all groups
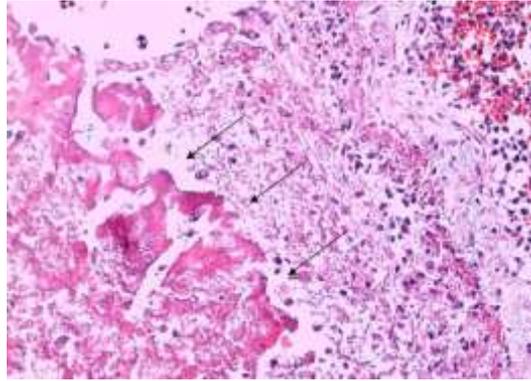
Fig-4: Histopathological view of a section from the graft + resveratrol (GR) group on 28th day. New bone formation (black arrows), Haematoxylin-eosin x200

Fig-5: Histopathological view of a section from the graft + resveratrol (GR) group on 14th day. Increased vascularization (black arrows), Haematoxylin-eosin x200
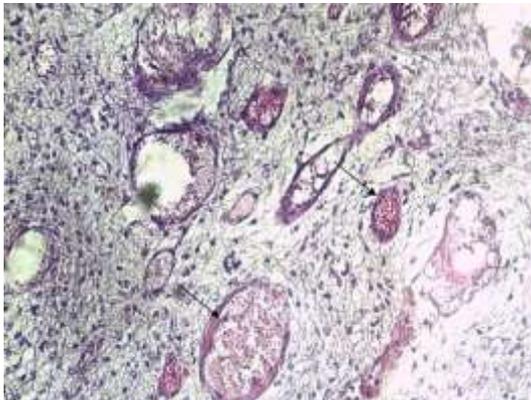

Fig-6: Histopathological view of a section from the graft + resveratrol (GR) group on 28th day. Increased vascularization (black arrows), Haematoxylin-eosin x360
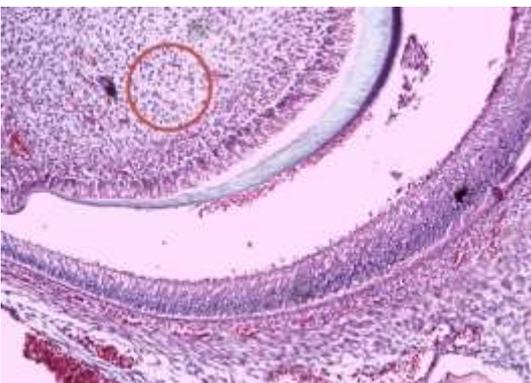

Fig-7: Histopathological view of a section from the graft + resveratrol (GR) group on 14th day. Fibrosis (red circle), Haematoxylin-eosin x140

DISCUSSION

The use of herbal and natural products with antioxidant properties has gained popularity in the recent years, and thus, these products have been recognized worldwide as alternative treatments. Systemic or local administration of several antioxidant substances or growth factors stimulates the formation of new bones.

Resveratrol is a polyphenolic phytoalexin. Phytoalexins are chemical substances, which are produced by plants as a part of the defense mechanism against pathogenic microorganisms or external stresses. Resveratrol is present in a traditional plant in Japan called kojo-kon that has been used for many years [10]. We administered resveratrol systemically to accelerate bone healing and to allow the bone graft to rapidly integrate with the host bone with few complications due to the antioxidant properties of the drug.

Although the bone tissue has a high repair ability, complete regeneration depends on the size of the defect. A critical-sized defect is the smallest dimension of an intraosseous wound that does not heal spontaneously during the lifetime of the individual [11]. A consensus has not been established to date about the dimensions of the critical-sized defect. The critical-sized defect in rats is generally a 4-5-mm defect in the calvarium. Nyan *et al.*, [12], Calixto *et al.*, [13] and Lima *et al.*, [14] examined a critical-sized defect of the rat parietal bone measuring 5 mm in diameter. The smallest critical-sized defect in rats was a defect measuring 4 mm in diameter at the mandibular angle. Recent studies have established a defect measuring 5 mm in diameter at the head and neck region of rats as an accepted critical-sized defect [15]. We created a 5-mm defect in the rat mandible.

Although autogenous bone grafts are still considered to be the gold standard for the treatment of defects that fail to heal spontaneously, these grafts are associated with some disadvantages, and thus, other types of grafts (allografts, xenografts, and alloplastic grafts) have been used. Animal-derived xenografts are appropriate alternative materials for bone regeneration. Previous studies using deproteinized bovine bone grafts show that these materials are biocompatible and osteoconductive, are not associated with immunological or allergic reactions, and are successfully used for filling bone defects and in sinus augmentation procedure [16-19]. Thus, we used deproteinized bovine bone grafts to fill the critical-sized bone defects in our study.

We performed histopathological examination on days different from those reported in the previous studies. Typically, histopathological examination of bone healing is assessed on the 3rd, 7th, 14th, 21th, and 28th days and 2nd or 3rd months after placement of the graft [13, 20, 21]. We performed histopathological examination to assess bone healing after sacrificing the animals on the 14th and 28th day.

A previous study indicates that resveratrol is used at doses ranging from 2.5 to 100 mg/kg [19]. The results of clinical and laboratory studies show that resveratrol has no known side effects such as mutagenicity, carcinogenicity, cytotoxicity, or allergic reactions [10]. Elmalı *et al.*, [7] and Casarin *et al.*, [8] administered resveratrol to experimental animals at a dose of 10 mg/kg per day via oral gavage. We administered resveratrol systemically at a dose of 10 mg/kg via oral gavage, according to the protocol described in previous studies.

Antioxidant substances are known to have favorable effects on bone healing. Several in vitro studies have shown the positive effects of resveratrol on cell proliferation [22, 23]; to date, however, a limited number of in vivo studies have investigated the effects of resveratrol on bone healing. An in vivo study by Mizutani *et al.*, [9] shows that resveratrol inhibits bone loss in ovariectomized rats. Casarin *et al.*, [8] created critical-sized defects of 5 mm in rat calvarias. They administered 10 mg/kg resveratrol per day via oral gavage. After 30 days, histomorphometric examinations of the control and experimental groups showed that the size of the defect was smaller in the resveratrol group than in the control group.

We examined bone healing by performing histopathological examination. Compared to the control group, the R and the GR groups showed an increase in ossification on days 14 and 28. The degree of ossification in the GR group was significantly high on the 28th day.

Resveratrol plays a unique role in physiological angiogenesis. The results of a study investigating the effects of resveratrol on incisional wound healing in rats showed that rats in the resveratrol group showed better vascularity than those in the control group [24]. Our results showed that rats in the GR group had greater vascularization than those in the other groups on the 14th and 28th days.

Resveratrol exerts anti-inflammatory effect by inhibiting free radicals derived from neutrophils, monocytes, and macrophages [25]. In addition, resveratrol inhibits the release of various cytokines from macrophages and lymphocytes [26]. Our results showed that the density of inflammatory cells and hyperemia on day 14 were significantly lower in the GR group than in the other groups. No significant difference was observed in these parameters between the groups on the 28th day.

During the early phase of bone healing, migration of fibroblasts to the affected area increases collagen synthesis, but prolonged fibroblast has a negative effect on bone healing [27]. Compared to the control group, the GR group showed a significant increase in fibrosis on day 14; however, no significant difference was observed in fibrosis between the groups on the 28th day.

BMPs are growth and differentiation factors that belong to the transforming growth factor-β (TGF-β) superfamily. BMP-2 is the most abundant osteoinductive protein among the BMPs [28]. BMP-2 promotes new bone formation by affecting the differentiation and proliferation functions of osteoblasts and chondrocytes [29]. Su JL *et al.*, [30] administered 10 mg/kg resveratrol to ovariectomized rats and determined the serum levels of BMP-2 using ELISA; the results of their study showed that the BMP-2 levels were higher in the experimental group than in the control group.

Compared to the control group, the R and the GR groups showed a significant increase in the levels of BMP-2 on the 28th and 14th days, respectively. Thus, our results indicate that systemic administration of resveratrol increases the levels of BMP-2. In addition to the high BMP-2 levels, high levels of ossification were observed on histopathological examination in the GR group, which supports our hypothesis that resveratrol may contribute to bone healing.

Resveratrol exerts anti-inflammatory effects by inhibiting cyclooxygenase and lipoxygenase enzymes, inhibiting the expression of certain inflammatory cytokines (TNF-α, interleukin 1β [IL-1β], and IL-6), inhibiting nuclear factor kappa B (NF-kB), and decreasing the expression of endothelial adhesion molecules [25]. Our results showed that the levels of TNF-α were lower in the groups receiving resveratrol than in the other groups on the 14th and 28th days. Histopathological examination showed low levels of inflammatory cells in the groups receiving resveratrol, which was consistent with the low levels of TNF-α; thus, our results indicate that resveratrol decreases the severity of inflammation.

CONCLUSIONS
Our results showed that systemic administration of resveratrol had beneficial effects on bone healing, had increased anti-inflammatory effects, and promoted vascularization in rats.

Moreover, systemic resveratrol in combination with a graft was effective in healing of critical-sized defects. Our results indicate that resveratrol promotes bone healing, and our findings contribute to the existing data about these effects of resveratrol.

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