

Effects of resveratrol and cigarette smoking on bone healing: histomorphometric evaluation

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Background/aim: The aim of the present study is to investigate the effects of cigarette smoke (CS) and simultaneous application of resveratrol on bone healing histomorphometrically and to evaluate the effects of resveratrol on negative effects of CS.

Materials and methods: For 4 weeks, Sprague Dawley rats were exposed to cigarette smoke at the equivalent of 6 cigarettes per day. After this period, monocortical defects were created in femurs by a trephine bur on day 28. Starting from the day of defect creation to the 28th postoperative day, rats were given 20 mg/kg body weight resveratrol. Histomorphometric examination of the number of osteoblasts and osteoclasts, as well as new bone area, was conducted.

Results: Investigations were carried out on 33 rats. Differences between osteoblast numbers in the control and CS groups were significant, and CS caused a reduction in the number of osteoblasts. Areas of new bone formation in the resveratrol and control groups were higher than in the smoking and smoking+resveratrol groups.

Conclusion: Smoking appeared to have adverse effects upon bone healing and resveratrol administration helped to reduce these effects.

Key words: Cigarette smoking, resveratrol, new bone area, osteoblast, osteoclast

1. Introduction

Bone healing is a complex process influenced by biological, mechanical, and systemic factors. Delays in bone healing affect the success of almost all oral surgical procedures. Bone healing may be decelerated for various reasons or may result in formation of fibrous tissue (1).

Cigarette smoke (CS) disrupts bone healing, can lead to osteoporosis, and affects the planning and success of surgical procedures. The effect of CS on bone metabolism could be via disorders in bone angiogenesis, collagen metabolism, and the RANK–RANKL–osteoprotegerin (OPG) system (2,3). Direct cellular effects of CS on bone include changes in calciotropic hormone metabolism (4). Osteocalcin can be affected by CS directly or by hormonal changes and may decrease osteoblast activity (5). Fung et al. (6) reported that nicotine (active ingredient of cigarettes) reduced vitamin D storage and osteoblast activity in humans.

Resveratrol (trans-3,4,5-trihydroxystilbene), a natural polyphenol, is a plant metabolite found in grapes and wine, with osteogenic, antioxidant, antiinflammatory, analgesic, and antiangi effects (7–9). In vitro studies have shown

that resveratrol inhibits osteoclast differentiation, increases formation of osteoblasts, and stimulates expression of the vitamin D receptor in bone marrow osteoblast precursor cells (10,11). Studies using various vitamins and hormones to reduce or eliminate the negative effects of CS on the healing and metabolism of bone have been published (12,13). However, these substances affect only osteoblasts or osteoclasts.

The aim of the present study is to investigate the effects of CS and simultaneous application of resveratrol on bone healing histomorphometrically and to evaluate the effects of resveratrol on negative effects of CS.

2. Materials and methods

2.1. Study design

Thirty-eight Sprague Dawley rats (6 months; 250–300 g) were obtained from the Experimental Animal Production and Experimental Research Laboratory of Süleyman Demirel University. The experiment was performed with the approval of the Ethics Committee on Animal Experiments of Süleyman Demirel University (date and number, 14.08.2012-10). Rats were placed in plastic cages

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according to standard conditions (21–22 °C; humidity, 50%; 12-h light/dark cycle). They were fed a standard pellet diet and tap water. Rats were divided randomly into four groups.

In the first 4 weeks of the study, CS exposure groups (groups 2 and 4) were exposed to cigarettes (Tekel 2000; İstanbul, Turkey) containing tar (10 mg), nicotine (1 mg), and carbon monoxide (10 mg) in glass cages of dimensions of 75 × 75 × 50 cm. Nonsmoking rats were exposed to room air in separate glass cages so that they experienced the same level of stress.

CS was produced in a glass smoke generator with an open bottom fitted to a cap covering the chamber. Cigarettes were burned in a filter and spilling of ash into the cage was not possible. The smoke generator was a reservoir with a 2-cm² opening at the top and dimensions of 25 × 15 × 15 cm. CS was produced by the burning of one cigarette/10 min in the open-base chamber. Smoke arising from the filter and the end of the cigarette was aspirated into the cage with the help of the fan fixed on the glass cover. All smoke in the upper part of the smoke generator was aspirated by a fan placed in the opening of the glass chamber (Figure 1). The CS experimental model was designed by the authors. The glass cage was ventilated for 5 min after each cigarette was burned. There were two sessions every day, and rats were exposed to the smoke of six cigarettes.

2.2. Surgical procedures

General anesthesia was induced with an intraperitoneal injection of 80 mg/kg body weight ketamine hydrochloride (Alpha®; Aegean-Vet, Turia, Spain). The surgical area was shaved and skin disinfected with a 10% povidone iodine solution. Under sterile conditions, a skin incision of about 1 cm in length was made with blunt dissection. The periosteum was elevated and reached the surface of the right femur. Using a standard trephine bur under

irrigation with 0.9% (physiological) saline, a monocortical defect (diameter: 3 mm) was osteotomized from the femur. Amikacin sulfate (5 mg/kg body weight daily, i.m.; Amikozit®, Zentiva, Turkey) was given for infection control, and ketoprofen (3 mg/kg body weight daily, i.m.; Profenid® bulb, Sanofi-Aventis, İstanbul, Turkey) was administered for pain control for 5 days after surgery.

According to the manufacturer's recommendations, resveratrol (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a ratio of 16 mg resveratrol/1 mL DMSO. Resveratrol solution was prepared fresh every day. After drawing resveratrol solution (20 mg/kg) into an insulin syringe, 1 mL of water was also drawn and administered by intragastric gavage to groups 3 and 4 for 4 weeks during the bone healing period, after surgery. Groups 1 and 2 were given only DMSO (diluted). CS exposure was continued during the bone healing period, after surgery. Rats were sacrificed at week 8. Right femurs were dissected and stored in 10% formalin.

2.3. Histomorphometric evaluation

Samples prepared for histomorphometric evaluation were stained with hematoxylin and eosin (H&E) and assessed using an Eclipse E400 Light Microscope (Nikon, Tokyo, Japan). A Coolpix 5000 Digital Camera (Nikon) was connected to the light microscope and samples were photographed during evaluation. Photographs taken at the same magnification with an MBM11100 Stage Micrometer Type A (Nikon) were recorded as digital images. All images were transferred to a personal computer and evaluated with Vision Lite 3.5 Image Analysis software (Clemex Technologies, Longueuil, Canada). In each case, osteoblasts and osteoclasts in an area of 562,375.2 µm² were marked with the image analysis system and counted automatically (Figure 2). On the same unit areas, formation of new bone was calculated by Vision Lite 3.5



Figure 1. The experimental cigarette smoking model.

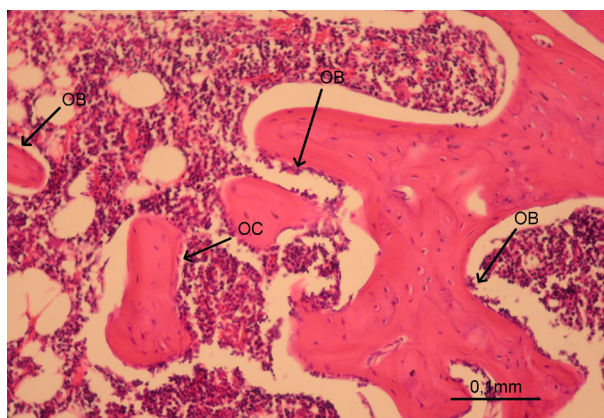


Figure 2. Histomorphometric evaluation, osteoblasts (OB) and osteoclasts (OC) (H&E).

Image Analysis. All histomorphometric analyses were undertaken by the same pathologist, who was blinded to the study protocol.

2.4. Statistical analyses

Statistical analyses were carried out using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Numbers of osteoblasts and osteoclasts were evaluated using one-way analysis of variance. Differences between groups were evaluated with the Tukey test. The Kruskal–Wallis test was used to evaluate the area of new bone formation. The Bonferroni–Dunn test was employed to determine differences in mean values between groups. $P \leq 0.05$ was considered significant.

3. Results

Of 38 rats, two died during the induction of anesthesia, two died due to the formation of perforations after intragastric gavage, and one was excluded from the study due to femur fracture of the defect line. Hence, investigations were carried out on 33 animals. No complications, osteomyelitis, abscess formation, or wound openings were seen after the surgical procedure to the time of euthanasia.

The highest number of osteoblasts was in the resveratrol group, and the difference between CS groups was significant. The differences between the osteoblast numbers of the control and CS groups were significant, and CS caused a reduction in the number of osteoblasts (Table 1).

Differences between the number of osteoclasts in the control group and CS group were significant ($P < 0.05$). The largest number of osteoclasts was in the control group, followed by the resveratrol group and CS+resveratrol group. The lowest number of osteoclasts was in the CS group (Table 2).

Data on new bone formation were transformed logarithmically and provided the preconditions of parametric testing. The results of the Kruskal–Wallis test at $P < 0.05$ indicated no significant difference between average values of the groups ($P = 0.074$), but the largest area of new bone was in the resveratrol group. Areas of new bone formation in the resveratrol and control groups were higher than in the CS and CS+resveratrol groups (Table 3).

Table 1. The results of osteoblast numbers between study groups.

Group	N	Mean	St Dev	Min	Max
C ¹	8	57.88 ^{ab}	15.33	42.0	87.0
CS ²	9	38.11 ^c	9.6	22.0	51.0
R ³	8	75.25 ^a	24.89	44.0	119.0
CS+R ⁴	8	48.88 ^{bc}	10.11	37.0	71.0

¹C, control; ²CS, cigarette smoke; ³R, resveratrol; ⁴CS+R, cigarette smoke and resveratrol. Differences between groups were evaluated with the Tukey test. Statistical differences are indicated with superscript letters. Statistically significant differences between the groups and osteoblast numbers are stated with different letters ($P < 0.05$).

Table 2. The results of osteoclast numbers between study groups.

Group	N	Mean	St Dev	Min	Max
C ¹	8	4.000 ^a	1.927	2.0	8.0
CS ²	9	2.444 ^b	0.726	1.0	3.0
R ³	8	3.750 ^{ab}	1.581	2.0	7.0
CS+R ⁴	8	2.625 ^{ab}	0.916	1.0	4.0

¹C, control; ²CS, cigarette smoke; ³R, resveratrol; ⁴CS+R, cigarette smoke and resveratrol. Differences between groups were evaluated with the Tukey test. Statistical differences are indicated with superscript letters. Statistically significant differences between the groups and osteoblast numbers are stated with different letters ($P < 0.05$).

Table 3. The results of new bone area between study groups.

Group	N	Mean	Mean Rank	St Dev	Min	Max
C ¹	8	1,429,279	21.0	605,549	532,283	2,377,681
CS ²	9	847,179	11.9	743,041	42,348	2,460,250
R ³	8	1,506,195	22.0	746,670	485,277	2,716,080
CS+R ⁴	8	884,297	13.8	215,486	664,151	1,266,354

¹C, control; ²CS, cigarette smoke; ³R, resveratrol; ⁴CS+R, cigarette smoke and resveratrol.

The Kruskal–Wallis test was used to evaluate the area of new bone formation. There is no statistical difference between groups and new bone area (P = 0.074).

4. Discussion

Bone healing after surgical procedures results in the achievement of bone structure and biomechanical strength. Bone healing is based on osteoblast differentiation into osteogenic cells as well as their proliferation and migration (14). CS influences bone metabolism by disrupting osteoblast formation and increasing osteoclast resorption (5,15).

Studies on bone healing have suggested that 4 weeks is an appropriate period to observe osteoblasts, osteoclasts, and new bone formation (16,17). Bone healing evaluated in the 4-week period suggests that osteoblasts are active and that new bone remodeling occurs in the defect. Maxillofacial surgical procedures performed in rats can cause severe malnutrition and general weakness. Histing et al. (18) reported that because of the easier surgical manipulation of the long bones, the femur or tibia can be preferred as a bone healing model.

Nicotine changes bone histology by inhibiting osteoblastic cells and disrupting PTH levels, causing tissue ischemia with increased platelet adhesion and microvascular congestion, decreasing bone density, and decreasing storage of vitamin D (6). Cadmium can also decrease bone density (19). Fracture healing and bone-graft recovery is disrupted in cigarette smokers and, in healed bones, tensile strength is decreased significantly (20–22). CS influences bone cells directly and is detrimental to bone formation because it inhibits osteoblast differentiation (1,23).

It has been suggested that aryl hydrocarbons in cigarettes might influence bone metabolism. Aryl hydrocarbons inhibit osteoblast differentiation and induce osteoclastic bone resorption. Resveratrol is a strong aryl hydrocarbon receptor antagonist and might reduce the inhibition of osteoblastogenesis and osteoclast formation caused by aryl hydrocarbons (15,23).

In addition to the antioxidant, antiinflammatory, antiaging, and anticarcinogenic characteristics of resveratrol, it is a plant metabolite with positive influences

on bone metabolism (7–9). Studies have shown that resveratrol stimulates expression of the vitamin D receptor in bone marrow cells, increases osteoblast differentiation while inhibiting osteoclasts, and accelerates bone healing (10,11,24). It has been reported that the antiinflammatory effect of resveratrol prevents the bone resorption caused by the increase in osteoclast activation related to oversecretion of inflammatory mediators (25).

In the present study, after forming a subchronic CS model, resveratrol was given at 20 mg/kg body weight daily over 28 days. Numbers of osteoblasts and osteoclasts, as well as new bone area, were evaluated. The number of osteoblasts was greatest in the resveratrol group and lowest in the smoking group (P < 0.05). Our research shows that resveratrol increases osteoblast formation. This result is in accordance with those of Liu et al. (1) and Boissy et al. (10) showing that resveratrol increases osteoblastogenesis in vitro. In addition, it could be thought that resveratrol “corrected” the decrease in the number of osteoblasts in the CS+resveratrol group.

The largest number of osteoclasts was in the control group, followed by the resveratrol group and the CS+resveratrol group. The lowest number of osteoclasts was in the CS group. Moreover, the number of osteoclasts in the resveratrol group was lower than in the control group. These results could be related to the inhibition of osteoclasts by resveratrol and are in accordance with the work of Boissy et al. (10) and Iqbal et al. (15).

In vitro and in vivo studies have evaluated the effects of resveratrol on bone formation. Those investigations suggested that resveratrol increased osteoblastogenesis and new bone formation (26,27). CS has been reported to cause hormonal changes, oversecretion of inflammatory cytokines, changes in levels of growth factors, and OPG inhibition. As a result, the balance between osteoblasts and osteoclasts is disrupted and, eventually, this might reduce new bone formation (28,29). Uysal et al. (30) injected a single dose of resveratrol (10 µmol/kg) into the maxillary suture of rats, to which intermaxillary suture extension

was conducted. They found that the new bone area and osteoblast numbers in the resveratrol-injected group were significantly greater than those of the control group.

Despite the absence of a significant difference ($P = 0.07$) between groups, new bone area was greatest in the resveratrol group and least in the CS group. This result supports the results of Andrade et al. (14), who found that CS diminished the bone volume of rats exposed to six cigarettes per day over 10 weeks.

Thus far, no in vivo study has been conducted to investigate the effects of both CS and resveratrol on bone healing, and the effects of resveratrol and CS on bone cells have been described in independent in vitro studies. In this in vivo study conducted in accordance with previous

studies performed at the cellular level, simultaneous application of resveratrol may have stimulated bone formation and it could be thought that resveratrol reduces the adverse effects of smoking on bone healing. Further biochemical and clinical studies are necessary to validate its effects on smoking patients and also to ascertain whether it should be used prophylactically.

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