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A comparative investigation of the effects of Resveratrol and dental pulp delivered mesenchimal stem cells on rat tibia bone defect healing

Una investigación comparativa de los efectos del Resveratrol y la pulpa dental proporcionó células madre mesenquimales en la curación de defectos óseos de la tibia de rata

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ABSTRACT

Resveratrol (3,4,5–trihydroxystilbene), an antioxidant compound, has a natural phytoalexin structure and also has many properties such as anti–inflammatory, antineoplastic and antiplatelet. In addition, mesenchymal stem cells isolated from various tissues are considered as a potential cell source for bone regenerative therapies. The present study aims to examine the effects of Resveratrol and dental pulp–derived mesenchymal stem cells on new bone formation in rats, both isolated and combined, by immunohistochemical methods. Twenty eigth Spraque Dawley male rats were used in the study. The rats were divided into four groups with seven rats in each group; the control group (Group 1)(n=7), the Systemic Resveratrol group (Group 2)(n=7), the Stem cell group (Group 3)(n=7), the Stem cell + Systemic Resveratrol group (Group 4)(n=7). A defect was opened on the tibia bones of the rats in all groups with a trephane bur (diameter of 3 mm and a length of 4 mm). After the 4–week experiment, all rats were sacrificed following the experimental protocols specific to each group. The specimens of tibia were subjected to histomorphological examination in fixative solutions. Values of inflammation, connective tissue formation, osteoclastic activity, osteoblast values, new bone formation, BMP2 and BMP4 expression levels obtained for all groups were evaluated by statistical analysis. Compared to the control group, new bone formation and osteoblastic activity were found to be significantly higher in the Stem cell group and Stem cell + Systemic Resveratrol group. (P=0.001) Additionally, new bone formation in the Systemic Resveratrol group was found to be significantly lower than in the Stem cell + Systemic Resveratrol group. (P=0.006) No significant difference was observed between other groups. (P>0.05) According to the results of the study, it was observed that Stem cell + Resveratrol treatment was more effective than isolated Resveratrol or isolated stem cell treatment applications, it induced the development of more bone trabeculae, decrease inflammation and increased the number of osteoblasts involved in bone formation. In the light of these data, it was concluded that the combined use of Resveratrol and Stem cells is more effective on the healing of bone defects than their isolated use.

Key words: Bone defect; Resveratrol; mesenchymal stem cell; rat; experimental study

RESUMEN

El Resveratrol (3,4,5–trihidroxiestilbeno), un compuesto antioxidante, tiene una estructura de fitoalexina natural y además tiene muchas propiedades tales como antiinflamatoria, antineoplásica y antiagregante plaquetaria. Asi mismo, las células madre mesenquimales aisladas de diversos tejidos se consideran una fuente potencial de células para terapias regenerativas óseas. El presente estudio tuvo como objetivo examinar los efectos del Resveratrol y las células madre mesenquimales derivadas de la pulpa dental sobre la formación de hueso nuevo en ratas, tanto de forma aisladas como combinadas; evaluadas mediante métodos inmunohistoquímicos. En el estudio se utilizaron 28 ratas macho Spraque Dawley. Las ratas se dividieron en cuatro grupos con siete ratas en cada grupo; el grupo de control (Grupo 1)(n=7), el grupo de Resveratrol sistémico (Grupo 2)(n=7), el grupo de células madre (Grupo 3)(n=7), el grupo de células madre + Resveratrol sistémico (Grupo 4)(n=7). Se creo un defecto en los huesos de la tibia de las ratas de todos los grupos con una fresa de trefano (diámetro de 3 mm y una longitud de 4 mm). Después del experimento de cuatro semanas, todas las ratas fueron sacrificadas siguiendo los protocolos experimentales específicos de cada grupo. Los tejidos provenientes tibias de animales sacrificados se sometieron a examen histomorfológico en soluciones fijadoras. Los valores de inflamación, formación de tejido conectivo, actividad osteoclástica, valores de osteoblastos, formación de hueso nuevo, niveles de expresión de BMP2 y BMP4 obtenidos para todos los grupos se evaluaron mediante análisis estadístico. En comparación con el grupo de control, se observó que la formación de hueso nuevo y la actividad osteoblástica eran significativamente mayores en el grupo de células madre y en el grupo de células madre + Resveratrol sistémico. (P=0,001) Además, se encontró que la formación de hueso nuevo en el grupo de Resveratrol sistémico era significativamente menor que en el grupo de células madre + Resveratrol sistémico (P=0,006) No se observaron diferencias significativas entre otros grupos (P>0,05). Según los resultados del estudio, se observó que el tratamiento con células madre + Resveratrol era mucho más efectivo que las aplicaciones de tratamiento con Resveratrol aislado o células madre aisladas, ya que, inducía el desarrollo de más trabéculas óseas, suprimía más la inflamación y aumentaba el número de osteoblastos involucrados. En la formación ósea. A la luz de estos datos, se concluyó que el uso combinado de Resveratrol y células madre es más eficaz en la curación de defectos óseos que su uso aislado.

Palabras clave: Células madre mesenquimales; defecto óseo; rata; Resveratrol; estudio experimental

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INTRODUCTION

In the treatment of defects resulting from previous operations in oral and maxillofacial reconstructive surgery, congenital defects and especially trauma, the lost bone tissue must be reconstructed [1]. In order to accelerate healing after bone defects, mechanical methods such as electrical and electromagnetic therapy, hyperbaric oxygen therapy, ultrasound application and low-level laser therapy (LLLT) are applied. Local/systemic medications can be applied as chemical methods [2]. Nowadays, especially since chemical methods can have many side effects, alternative methods with fewer side effects and similar benefits have begun to be used in studies instead of these agents.

The use of antioxidant agents and compounds obtained from plants has become widespread. Resveratrol (RSVL, 3, 4', 5 trihydroxystilbene), one of these compounds, has become important for use in clinical and pharmacological studies [3]. RSVL is a compound with antioxidant properties in the natural phytoalexin structure, which is found in high amounts in vegetables and fruits such as grapes, peanuts, raspberries, mulberries, plums, blackberries; red wine; and the roots of the plant called *Polygonum Cuspidatum* [4]. *In vitro* and animal studies have shown that RSVL has biological activities such as anti-inflammatory, anti-arthritic, anti-carcinogenic, anti-diabetic, anti-oxidant, anti-aging effects and estrogenic activity [5]. *In vitro* studies have shown that RSVL treatment prevents osteoclastogenesis while activating osteoblastogenesis on bone metabolism [6, 7].

Stem cells are important in bone healing due to their ability to self-renew and differentiate into various cells [8]. In particular, mesenchymal stem cells (MSCs) isolated from different tissues are used as a potential cell source for cell replacement and bone regenerative therapies, in addition to their osteoblastic activities [9, 10]. Although MSCs are obtained from many different sources, stem cells from dental pulp have advantages such as being easily obtained without the need for a different surgical procedure and having a very rich stem cell population, compared to stem cells from bone marrow and other tissues [11]. It has been shown that stem cells obtained from dental pulp (DPSC) can transform into odontoblast, myocyte, osteoblast, chondrocyte, adipocyte and corneal epithelial cells [12, 13].

The present study aims to examine the effects of RSVL and DPSC on new bone formation in rats (*Rattus norvegicus*), both isolated and combined, by immunohistochemical methods.

MATERIALS AND METHODS

Ethics and animals

All surgical and experimental procedures were performed at the Dicle University Experimental Research Center. The content of the study was approved at the Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research and Application Center Animal Experiments Local Ethics Committee with the date 26.02.2020, protocol 01/2020 and number 02. The study was conducted in accordance with the principles of the Declaration of Helsinki for the protection of laboratory animals. 28 male Sprague-Dawley rats (3-5 months old, 250-300 g) were obtained from the Dicle University Experimental Animal Production and Research Center and experimental procedures were carried out in the same center. Obtaining stem cells was carried out at Muğla Sıtkı Kocman University, Department of Genetics and Bioengineering. The selected animals were kept in plastic cages in a room with a temperature of 21±10°C. During the experiment, the rats were kept in an environment that provided a 40-60% humidity standard with a 12-hour daylight cycle. After the experiment, seven subjects were kept in the same metal cage. Experimental animals were fed with standard rat chow containing 21% protein and tap water.

Study design

The 28 male Sprague-Dawley rats were randomly divided into four groups of seven. Block randomization procedure was not used to assign rats to different groups. For standardization, a homogeneous experimental pool was created by looking at the weight and age of all rats. Randomization sequence concealment and masking was taken into consideration when grouping the rats.

1. **Control Group (Group 1):** The defect area (diameter of 3 mm and a length of 4 mm) created in the tibia of the rats was allowed to heal on its own. The rats were kept under observation for four weeks and then sacrificed (n=7).
2. **Systemic Resveratrol Group (Group 2):** A defect (diameter of 3 mm and a length of 4 mm) was created in the rat tibias and RSVL was given systemically via oral gavage at 10 $mg \cdot kg^{-1}$ every day for four weeks. The rats were observed for four weeks and then sacrificed (n=7).
3. **Stem cell Group (Group 3):** After creating a defect (diameter of 3 mm and a length of 4 mm) in the rat tibia and suturing the periosteum and skin, the rats to which DPSC were applied locally were kept under observation for four weeks and then sacrificed (n=7).
4. **Stem cell + Systemic Resveratrol Group (Group 4):** After creating a defect (diameter of 3 mm and a length of 4 mm) in the rat tibia and suturing the periosteum and skin, the rats were administered DPSC locally and RSVL was administered systemically via oral gavage at 10 $mg \cdot kg^{-1}$ every day for four weeks, and were kept under observation for four weeks and then sacrificed. (n=7).

Preparation and dosage of Resveratrol

RSVL (Sigma, catalog number: R5010, USA) was weighed at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for each rat. RSVL was dissolved in ethanol and diluted with physiological saline (1:3) and the solution was prepared daily. Starting from the day of operation, 10 $mg \cdot kg^{-1}$ RSVL solution per day was applied to the Resveratrol and Resveratrol + Stem cell applied rat groups by oral gavage method, once a day and at the same time every day.

Obtaining dental pulpa mesenchymal stem cells and way of application

Tooth extraction was performed from the lower molars of two adult male experimental animals of the Sprague Dawley lineage under anesthesia (Ketamine: 75-90 $mg \cdot kg^{-1}$ + xylazine 5-8 $mg \cdot kg^{-1}$, IP). To DPSC from extracted teeth, the teeth were cut into small pieces with a scalpel, then suspended in phosphate buffered saline (PBS) containing 0.1% collagenase and 0.25% trypsinethylenediaminetetraacetic acid and was incubated at 37°C for 30-60 min. DPSCs in the suspension passed through 70 mm cell filters were examined morphologically under a microscope (Soptop ICX41, Ningbo, China), and the cells were counted using a hemocytometer (Merck Bright-Line™, Darmstadt, Germany). 2×10^6 cells were planted in a 100 mm plastic petri dish. DPSCs were taken to the incubator for growth and incubated at 37°C, saturated humidity and 5% CO_2 conditions. The cells to be incubated were grown in alpha-modified Eagle medium containing 5.5 $mmol \cdot L^{-1}$ glucose, 20% fetal bovine serum and 1% penicillin streptomycin. When the cells reached 70-80% confluency, they were

passaged two more times and in the third passage (P3), the cells were characterized for use in experiments. The presence of MSCs was detected by FACS method with CD45 PC5, CD44 PE, CD105 FITC and STRO-1 PE antibodies. DPSC were expected to be CD44, CD105 and STRO-1 positive and CD45 negative. Since DPSC are capable of differentiation, osteogenic differentiation was tested. OriCellTM osteogenesis differentiation kit (Cyagen, Guangzhou, China) was used to induce osteogenic differentiation. The obtained stem cells were administered locally to the defect area with syringe applicator. The animals were returned to normal feeding and rearing conditions (fed with standard rat chow containing 21% protein and tap water).

Histologic study

All rats were sacrificed on the 28th day by applying 30 ml isoflurane (Isoflurane-USP, Adeka Ilac, Turkiye). The left tibia of the rats was removed and dissected. For histological analysis, samples were fixed in 10% neutral buffered formalin for 24 hours. Then, they were decalcified in 10% diluted formic acid at room temperature for one month and then embedded in paraffin blocks. With the help of a microtome (Catalog no: Leica RM2265, Wetzlar, Germany), 4-6 µm thick sections were taken from the blocks for Hematoxylin-Eosin and immunohistochemical staining. The sections were examined using a light microscope (Nikon Optiphot-2; Nikon Corporation, Tokyo, Japan) by researchers who were blind to the treatment assignments. Values of inflammation, connective tissue formation, osteoclastic activity, osteoblast values, new bone formation, Bone Morphogenic Protein 2 (BMP2) and Bone Morphogenic Protein 4 (BMP4) expression levels were determined semi-quantitatively scored.

Semi-quantitative Histologic Scoring

Scoring was performed using a 0 to 4 scoring system (0, no; 1, minimal; 2, moderate; 3, abundant).

Immunohistochemistry study

Sections taken from the paraffin blocks were placed in a double boiler set at 37°C and then onto polylysine slides. The sections were deparaffinized in xylene for 3×15 min. The sections were placed in an ethylenediamine tetraacetic acid (EDTA) buffer solution (pH: 8.0, catalog no: ab93680, Abcam, Cambridge, USA) and heat-induced epitope retrieval was performed.

Hydrogen peroxide 71 solution (catalog no: TA-015-HP, ThermoFischer, Fremont, CA, USA) was dropped onto the sections. Sections were processed by anti-BMP-2 (catalog no: sc-137087, Santa Cruz Biotechnology, 10410 Finnell St, Dallas, Texas 75220, US) and anti-BMP-4 (catalog no: sc-393329, Santa Cruz Biotechnology, 10410 Finnell St, Dallas, Texas 75220, US) overnight at +4°C with antibodies. Biotin secondary antibody (catalog no: TP-015-BN, ThermoFischer, Fremont, CA, USA) was dropped onto the sections washed with PBS and incubated for 14 min. Then, Streptavidin-peroxidase (catalog no: TS-015-HR, ThermoFischer, Fremont, CA, USA) was added and washed with PBS after waiting for 15 min. Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) was dropped onto the washed sections, the reaction was monitored under a microscope and stopped with PBS. After counterstaining with Harris hematoxylin, the sections were covered with Entellan (catalog no: 107961, Sigma-Aldrich, St. Louis, MO, USA) and evaluated and viewed with a Zeiss Imager A2 photomicroscope (Carl Zeiss, Jena, Germany).

Statistical analysis

While evaluating the findings from the study, SPSS for Windows version 24.0 package program was used for statistical analysis. While evaluating the study data, the suitability of the data to normal distribution was tested with the Shaphiro Wilk test, and Kruskal Wallis and Dunn Multiple Comparison tests were used to compare the features that did not comply with normal distribution in more than 2 independent groups. Significance was evaluated at $P<0.05$ level.

RESULTS AND DISCUSSION

FIG. 1 shows the alveolar bone sections of all groups that were stained with hematoxylin and eosin on days 28 after experimental. In Group 1, mild degenerations were observed in some osteocyte cells located especially close to the endosteal region in the compact bone region. It was determined that osteoclastic activity began to become particularly evident in areas close to this area (FIG. 1A). In Group 2, osteoblastic activity was observed to start especially in areas close to the endosteal area (red arrow). Osteocytes have not yet been observed very clearly in the endosteal region (FIG. 1B). In Group 3, it was observed that especially the newly formed bone trabeculae pieces, towards the outside of the endosteal region, were rich in osteoblasts (red arrow), started to develop and expand. A decrease in the number of osteoclasts was also detected (FIG. 1C). In Group 4, bone trabeculae of different sizes began to be seen in the defect area (black arrows). A increase in osteoblasts, especially in bone trabeculae (red arrow), a decrease in osteoclasts, and occasionally osteocyte cells with distinct lacunae (yellow arrows) were observed within the bone trabeculae. It was determined that the matrix increase was intense and the bone trabeculae widened (FIG. 1D).

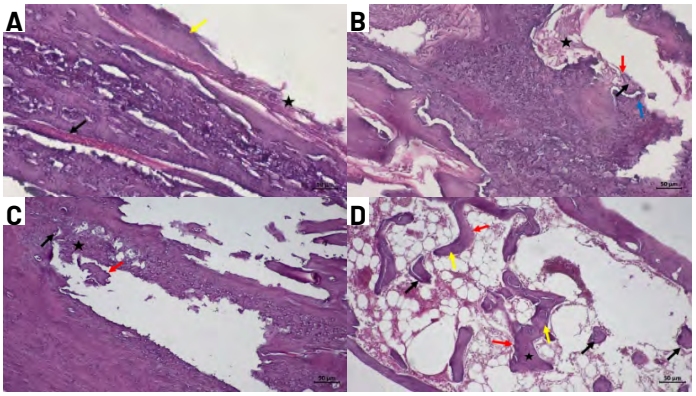

FIGURE 1. Defect area of the A) Control group (Bar scale: 50 µm), B) Systemic Resveratrol group (Bar scale: 50 µm), C) Stem cell group (Bar scale: 50 µm), D) Stem cell + systemic Resveratrol group (Bar scale: 50 µm) groups tibial bone in the fourth week view. (hematoxylin and eosin stain; magnification, 10x)

Immunohistochemistry results

BMP-2 (Bone Mineral Protein-2): In Group 1, in the longitudinal section of the tibial bone, there was a increase in inflammatory cells (asterisk), especially with an increase in dense connective tissue between the broken bone pieces, and intense osteoclastic activity (blue arrows), especially in the periphery of the bone trabeculae. showed a positive reaction with BMP-2 (FIG. 2A). In Group 2, it was observed that osteogenic activity turned in favor of bone formation, especially in the

osteoblast direction, BMP–2 showed a positive reaction in osteoblasts (red arrows), and osteocyte cells were not yet fully evident. While there was decrease in the number of osteoclast cells, an increase in BMP–2 activity was observed in the osteoclast cells here (blue arrows) (FIG. 2B). In Group 3, it was observed that bone trabeculae began to form in the tibial bone, especially in the area close to the actual bone where the defect area was located (black circle), osteoblastic activity increased, and a few osteocyte cells began to become evident. A decrease in osteoclast cells was observed (blue arrow).

The most important feature was that osteocytes in the new bone trabeculae clearly show the BMP–2 reaction and contribute to new bone formation (FIG. 2C). In Group 4, effect on ossification due to the occasional formation and expansion of new bone trabeculae, the intense expression of osteoblast cells (red arrows) in the periphery, the increase in matrix formation and the positive BMP–2 reaction of osteocytes (yellow arrows) was observed (FIG. 2D).

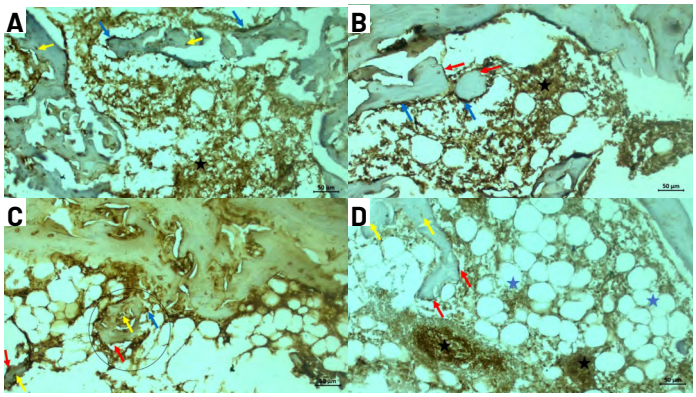

FIGURE 2. Defect area of the A) Control group (Bar scale: 50 µm), B) Systemic Resveratrol group (Bar scale: 50 µm), C) Stem cell group (Bar scale: 50 µm), D) Stem cell + systemic Resveratrol group (Bar scale: 50 µm) groups tibial bone in the fourth week view. (Immunohistochemical staining) (BMP–2)

BMP–4 (Bone Mineral Protein–4): In Group 1, BMP–4 expression was observed to be positive in some leukocytes (arrowhead), along with intense inflammatory cell infiltration around the vessel. Although there were signs of ossification in some areas, BMP–4 expression was seen at a high rate in osteoclast cells (blue arrows) due to the density of osteoclast cells (FIG. 3A). In Group 2, only osteoblastic (red arrows) activity was positive in small bone trabeculae. The BMP–4 reaction was evaluated as positive, with also a partial decrease in osteoclasts (blue arrows) (FIG. 3B). In Group 3, the BMP–4 reaction was observed to be positive, especially in osteoblasts (red arrow) and osteocytes (yellow arrows), which were enlarged within the defect area. BMP–4 reaction was also evaluated as positive in a small number of osteoclasts (blue arrow). Improvement was noted in new bone formation in terms of matrix development and lacunar structure (FIG. 3C). In Group 4, a mature bone trabecula located close to the endosteal region within the defect area is observed to begin to form (black circle). Osteoblasts located in the periphery were also seen to be prominent. BMP–4 reaction was found to be positive in osteoblasts (red arrow) and osteocyte cells (yellow arrow) (FIG. 3D).

According to TABLE I, there was no significant difference between the groups in terms of inflammation values (P=0.099). The fact that

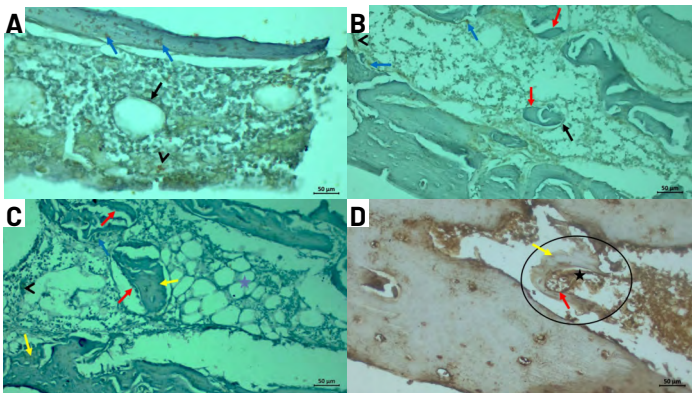

FIGURE 3. Defect area of the A) Control group (Bar scale: 50 µm), B) Systemic Resveratrol group (Bar scale: 50 µm), C) Stem cell group (Bar scale: 50 µm), D) Stem cell + systemic Resveratrol group (Bar scale: 50 µm) groups tibial bone in the fourth week view. (Immunohistochemical staining) (BMP–2)

TABLE I
Obtained for all groups; values of inflammation, connective tissue formation, osteoclastic activity, osteoblast values, new bone formation, BMP2 and BMP4 expression levels

	Control (n=7)	Stem Cell (n=7)	Resveratrol (n=7)	Stem Cell Resveratrol (n=7)	
Variables	**Median [25%–75%]**	**Median [25%–75%]**	**Median [25%–75%]**	**Median [25%–75%]**	***P***
Inflammation	3 (3 –4)	3 (2 –3)	3 (2 –3)	2 (2 –3)	0,099
Connective tissue formation	1 (0 –1)	2 (2 –2)	2 (2 –3)	3 (3 –3)	0,001*
Osteoclastic activity	3 (3 –4)	3 (2 –3)	3 (3 –3)	2 (2 –3)	0,043*
Osteoblastic activity	1 (1– 2)	2 (2 –2)	3 (2 –3)	3 (2 –3)	0,001*
New bone formation	0 (0 –0)	1 (1 –2)	2 (2 –3)	3 (3 –3)	0,001*
BMP–2	0 (0 –1)	2 (2 –3)	2 (2 –3)	3 (3 –3)	0,001*
BMP–4	0 (0 –1)	2 (2 –3)	2 (2 –2)	3 (3 –3)	0,001*

*: significant at 0.05 level; Kruskal Wallis test mean and standard deviation values

it was not significant in terms of inflammation may be due to the numerical difference between the groups.

For all other measurements, a significant difference was found in at least one group (P<0.05) (TABLE II). The analysis result regarding the difference between the groups in terms of osteoblast and new bone formation values is shown in TABLE II. Compared to the control group, new bone formation and osteoblastic activity were found to be significantly higher in Group 3 and Group 4 (P=0.001). Additionally, new bone formation was found to be significantly lower in Group 2 compared to Group 4 (P=0.006). No significant difference was observed between other groups (P>0.05) (TABLE II).

It was determined that there was a statistically significant difference between Group 2, Group 3 and Group 4 when compared with the Control group in terms of BMP–2 and BMP–4 values (P<0.05). In addition, the difference in the values of the 'Stem Cell' group (Group 3) in terms of BMP–4 was found to be significantly lower than the 'Stem Cell – Resveratrol' (Group 4) group (P<0.05) (TABLE III).

TABLE II
Analysis result regarding the difference between groups in terms of osteoblastic activity and new bone formation values

Group comparisons	Osteoblastic Activity	New Bone Formation
Group 1 / Group 2	0,110	0,101
Group 1 / Group 3	0,001*	0,001*
Group 1 / Group 4	0,001*	0,001*
Group 2 / Group 3	0,071	0,066
Group 2 / Group 4	0,071	0,006*
Group 3 / Group 4	1,000	0,366

*: significant at 0.05 level; Kruskal Wallis test and Dunn's multiple comparison test

TABLE III
Analysis result regarding the difference between groups in terms of BMP-2 and BMP-4 values

Group comparisons	BMP-2	BMP-4
Group 1 / Group 2	0,013*	0,004*
Group 1 / Group 3	0,005*	0,020*
Group 1 / Group 4	0,001*	0,001*
Group 2 / Group 3	0,718	0,587
Group 2 / Group 4	0,102	0,075
Group 3 / Group 4	0,203	0,020*

*: significant at 0.05 level; Kruskal Wallis test and Dunn's multiple comparison test

Since the oral and maxillofacial region is in constant function, it is constantly exposed to chemical and mechanical forces. Therefore, studies are being carried out on many factors that stimulate recovery in order to restore function to patients as soon as possible [14]. Especially today, systemic and local agents such as bisphosphonate and parathyroid hormone, which reduce resorption and increase deposition in bone, are preferred [15]. These agents used have many side effects. For this reason, alternative drugs with similar benefits but less potential for side effects have begun to be used in studies. While RSVL treatment stimulates osteoblastogenesis on bone metabolism, it also inhibits osteoclastogenesis [4, 6, 7]. In the current study, antioxidant RSVL was administered systemically to accelerate bone healing and increase stem cell activity.

The first *in vitro* study examining the effects of RSVL on the bone formation and destruction mechanism was conducted by Mizutani *et al.* [16] In this study, it was reported that RSVL activated the proliferation and differentiation of osteoblastic MC3T3-E1 cells *in vitro*. In a different study, it was reported that RSVL activates the proliferation and differentiation of osteoblast cells by increasing the expression of genes that have a supportive effect on osteogenesis, and that it acts on bone resorption by suppressing the activation of genes responsible for the formation of osteoclastogenesis [17, 18].

Mobasheri and Shakibaei concluded in their study where they evaluated the findings obtained from *in vitro* studies, that RSVL increased bone mass by activating bone formation while inhibiting bone resorption [6]. In the *in vitro* study conducted by Backesjo *et al.* [19], it was shown that RSVL increased osteoblast differentiation resulting in new bone formation and inhibited adipocyte formation and development. In the experimental study of rapid maxillary expansion conducted to examine the effectiveness of RSVL in local application by Uysal *et al.* [20], it was observed that the application of RSVL activated new bone formation and shortened the required retention time after the operation. Based on this, it is thought that RSVL can be used in the treatment of bone fractures and distraction osteogenesis. In this study, unlike the literature, it was observed that there was no significant difference in terms of new bone formation and osteoblastic activity when isolated use of RSVL was compared with the control group. In addition, it was observed that new bone formation and osteoblastic activity were significantly lower in the Stem cell + Resveratrol group than in the stem cell groups.

In their studies investigating the effectiveness of RSVL on the proliferation and differentiation of human bone marrow-derived MSCs, Ornstrup *et al.* [21] have shown that bone marrow-derived MSCs obtained from adult donors in long-term interaction with RSVL increased the osteoblastic activities. Although we used DPSC in our own study, our results are similar.

In the study where Song *et al.* [22] examined the effects of MSCs obtained from rat bone marrow on osteoblastic proliferation and differentiation with the use of RSVL and cyclosporine (CsA), the enhancing effect of RSVL on osteoblast cell differentiation and proliferation through the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling pathway, it has been shown that CsA has an inhibitory effect on osteoblastic proliferation and differentiation of MSCs obtained from rat bone marrow. In the present study, unlike the literature, no significant difference was found in osteoblastic proliferation and new bone formation in RSVL compared to the control group.

The effectiveness of RSVL on bone resorption was investigated by Liu *et al.* [17] in a rat model that underwent ovariectomy. Based on these findings, it was concluded that RSVL has an increasing effect on bone density and inhibits the loss of bone calcium amount. In addition, according to the findings, it is thought that RSVL may play a protective role against bone destruction caused by estrogen deficiency. In order to measure the effectiveness of RSVL on bone density in their study on ovariectomized rats, Lin *et al.* [23] have shown that daily RSVL intake activated bone formation in ovariectomized rats.

In the study conducted by Casarin *et al.* [3] to investigate the effectiveness of RSVL on bone defects, the aim was to evaluate the effects of RSVL application on the defect created in the calvarium and on bone healing around the titanium implant. As a result, it was reported that RSVL application increased critical size defect repair and biomechanical resistance of titanium implants, and also positively affected BMP-2, BMP-7 and osteopontin expression levels. Based on these results, we think that regular use of RSVL may be a useful supportive agent on the bone healing mechanism and in the treatment of edentulous individuals with dental implants. Similarly, in our study, it was determined that there was a significant difference in BMP-2 (P=0.013) and BMP-4 (P=0.004) expression values in the groups in which the critical size defect opened in the tibia in which RSVL was administered at a dose of 10 mg·kg^{-1} via oral gavage for 1 month, compared to the control group.

MSCs can be isolated from many tissues such as adipose tissue, umbilical cord blood, peripheral blood, dental pulp, dermis, amniotic fluid and even tumors. They can differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts and neurons [24, 25]. In particular, their osteogenic differentiation potential is superior to other stem cell types and therefore they are used to improve bone regeneration.

Stem cells obtained from dental pulp were first used clinically in 2009 by d'Aquino *et al.* [26] and were applied to 17 patients. According to the results of the study, it was observed that the stem cells taken from the patient were more satisfactory in terms of new bone height and tissue organization on the side where they were placed [26]. It has been shown in many studies that stem cells obtained from dental pulp can transform into osteoblasts under *in vitro* conditions with stimulation. There are even articles arguing that DPCH cells have greater differentiation ability under *in vitro* conditions than bone marrow stem cells, which are considered the standard in stem cell research [27].

In the study by Jing-hui *et al.* [28], it was reported that stem cells transplanted into a gelatin sponge could show osteogenic differentiation even in an ectopic location in mice (*Mus musculus*) and that DPCH cells could be used as a suitable and effective source in bone tissue engineering studies. DPCH is a rich resource that provides good results not only for the field of dentistry but also for other branches of medicine. Studies have shown that it can be used successfully in the treatment of myocardial infarction, nerve tissue regeneration, muscular dystrophy, cerebral ischemia, improvement of angiogenetic properties and corneal regeneration [29]. In our own study where we used DPCH, the results are consistent with the literature.

Two methods are frequently used for stem cell applications today. The first is intravenous injections (direct application of cells), the second is cell encapsulation systems (indirect application of cells with the help of a carrier) [30]. Accordingly, in our study, local transplantation of MSCs into the defect with the help of scaffolding was applied. The cell density of the MSCs to be used for tissue regeneration is also an important factor. After MSC application, sufficient tissue fluid and blood supply must be provided so that the cells can maintain their viability and differentiate into osteoblastic cells [31]. In studies using MSC concentrations between 2×10^6 – 2×10^7 cells·ml^{-1}, it has been reported that effective tissue regeneration occurs in various defects, including bone [32]. In the present study, MSCs at a density of 2×10^7 cells/ml, which is between these values, were used.

Looking at the literature, there are many studies examining the effects of systemically applied RSVL as a herbal agent and mesenchymal stem cell application, which is very promising in the field of molecular biology, on bone healing separately. However, there are very few studies comparing the effectiveness of RSVL in bone healing when used in combination with stem cells. Therefore, we think that the results of this study will contribute to the literature.

CONCLUSION

As a result of this study, it was observed that isolated RSVL and isolated DPSC application partially stimulated bone development after the defect and supported the development of new bone trabeculae. After the defect was created, it was observed that Stem cell + RSVL treatment was much more effective than isolated RSVL or isolated Stem cell treatment, it induced the development of more bone trabeculae, suppressed inflammation more, and increased the number of osteoblasts involved in bone formation.

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Conflict of interest

The authors declare that they have no conflicting interest.

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