Journal of Cranio-Maxillo-Facial Surgery 44 (2016) 1327–1332

Contents lists available at ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com

The influence of oral administration of rosuvastatin on calvarial bone healing in rats

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ARTICLE INFO

Article history:
Paper received 28 November 2015
Accepted 14 April 2016
Available online 22 April 2016

Keywords:
Rosuvastatin
Bone healing
Stereological
Micro CT

ABSTRACT

The purpose of this study is to investigate the potential of the systemic administration of different doses of rosuvastatin (RSV) on autogenous grafted critical-sized cortical bone defects. Twenty-four rats were divided into three groups: Group C (control), Group RSV-2 and Group RSV-5. A 5-mm diameter critical-size defect was created in the calvarium of each animal. In Group C, the defect was filled by autogenous graft only and rats were given saline solution with oral gavage for 28 days. In Group RSV-2 defects were filled with autogenous graft and rats were given 2 mg/kg rosuvastatin with oral gavage for 28 days. In Group RSV-5 defects were filled with autogenous graft and rats were given 5 mg/kg rosuvastatin with oral gavage for 28 days. All animals were euthanized at 28 days postoperative. Stereologic and micro-CT analyses were performed. New bone area (NBA) and connective tissue volumes were measured. Stereologic analysis showed that Group RSV-5 and RSV-2 had significantly more new bone at 4 weeks compared with group C. Connective tissue volumes were also significantly higher in RSV applicated groups. New bone and connective tissue volumes' difference were not statistically significant between RSV groups. Micro-CT results were similar with stereologic analyses. Orally administered RSV enhances bone regeneration in critical size calvarial rat defects filled with autogenous graft furthermore possible inflammatory effect should be investigated.

1. Introduction

Segmental or large bone defects may occur due to trauma, resection or pathology and present important clinical difficulty for oral and maxillofacial surgeons (Kneser et al., 2006). The treatment of extensive bone defects may require use of grafting procedure for optimum bone formation (Young et al., 2009). Bone substitutes have been used by clinicians for the reconstruction of osseous defects and autogenous bone is accepted as the gold standard (Becker et al., 1996). However, because of poor blood supply, scarred dura or insufficient soft-tissue coverage large craniofacial defects may be needed extra modalities to help increase the chance of successful treatment (Hopper et al., 2001).

Statins, 3-hydroxy 3-methyl glutaril coenzyme A (HMG-CoA) reductase inhibitors, were first developed to control and treat patients with hyperlipidemia and hypercholesterolemia and Mundy et al. (1999) reported beneficial effects of statins on osteoporotic patients. In recent years, it has been shown that statins also have positive role on bone formation by modulate inflammation, enhance osteogenesis and angiogenesis (Maeda et al., 2003; Hernández et al., 2014; Tan et al., 2015).

Statins increase the expression of important osteoanabolic and angiogenetic factors such as bone morphogenetic protein (BMP)-2 and vascular endothelial growth factor (VEGF) (Mundy et al., 1999; Maeda et al., 2003). BMPs are active bone-inducing factors that act on immature mesenchymal cells, including osteoblasts, resulting in osteogenesis and BMP-2 is the most potent of the osteoinductive factors (Wozeny et al., 1988). VEGF is an angiogenic cytokine and may induce proliferation and differentiation of osteoblasts by stimulating endothelial cells to produce osteoanabolic factors (Wozney, 1995; Wong and Rabie, 2005).

Rosuvastatin (RSV) is a lipid lowering drug used to prevent cardiovascular disorders. RSV has long terminal-life and powerful effect according to simvastatin and atorvastatin (Karlson et al., 2016). This drug also has pleiotropic effects, including bone

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http://dx.doi.org/10.1016/j.jcms.2016.04.021
1010-5182/

stimulation, promotion of vasculogenesis, and antiinflammatory effects (Monjo et al., 2010). Local effect of RSV on bone defects has been evaluated clinically and based on clinical parameters. (Pradeep et al., 2015a, b). Statins have extensive first metabolism in liver so they have low systemic bioavailability (Schachter, 2004). To investigate systemic effect of RSV on bone formation, higher dose of RSV should be used and tried on animal models to avoid side effects on human and find out right doses. Animal bone studies can be analyzed by histological methods but in clinical studies it may be an ethical problem to get a specimen from a patient for histological analyze. To the best of the authors' knowledge there is no study about systemic effect of RSV on bone defects.

The purpose of this study is to investigate the potential of the systemic administration of different doses of rosuvastatin (RSV) on autogenous grafted critical-sized cortical bone defects.

2. Materials and methods

2.1. Animals

Twenty-four 6-8 week-old Wistar rats were used. The animals were put in standard cages which were placed in rooms with a relative humidity rate of 40–60% and temperature of 22 ± 1 °C. The illumination system of the room was configured to automatically provide 12 h of day and 12 h of night and this study was approved by the Animal Experimentation Committee of Bülent Ecevit University, Zonguldak, Turkey.

2.2. Surgical procedure

All surgeries were performed under sterile conditions in an animal laboratory surgical suite. The rats were anesthetized by intramuscular injection of 3 mg/kg xylazine hydrochloride (Rompuns, Bayer, Leverkusen, Germany) and 35 mg/kg ketamine hydrochloride (10% Ketasol; Richter Pharma AG, Wels, Austria). After aseptic preparation, a semilunar incision was made and a full thickness flap was reflected exposing the parietal and frontal bones. A 5-mm diameter critical size calvarial defect was performed with a trephine used in a low-speed handpiece under continuous sterile saline irrigation. Care was taken during the surgery not to damage the dura mater. All defects were filled by autogenous grafts, the flap was sutured with resorbable 4/0 polyglactin 910 sutures (Vicryl; Ethicon, Somerville, NJ, USA). For postoperative infection control, 10 mg/kg cefazolin sodium (Sefazol; M Nevzat, Istanbul, Turkey) was injected to animals and metamizole sodium (Novalgin, Aventis, Turkey) as analgesic, for 5 days after the operation.

2.3. Experimental groups

Rats were divided into three groups of eight rats each:

Group C (Control): the defects were filled with autogenous graft and rats were given saline solution with oral gavage for 28 days.

Group RVS-2: the defects were filled with autogenous graft and rats were given 2 mg/kg rosuvastatin with oral gavage for 28 days.

Group RSV-5: the defects were filled with autogenous graft and rats were given 5 mg/kg rosuvastatin with oral gavage for 28 days.

RSV was pulverized to powder and dissolved in sterile distilled water.

Autogenous bone grafts were harvested from left tibia of rats. Medial surface of the left legs of the subjects were shaved and disinfected the area with povidone iodine solution. The legs were given the flexion position and longitudinal incisions of 20–25 mm were made periosteally in order to reach the medial surfaces of the tibia. The medial surfaces of the tibia were exposed with blunt dissection and soft tissues were excluded. Autogenous bone graft, covering the cortex and medulla layers of the bone was obtained by using round-tipped, stainless steel drill with a diameter of 3 mm under sterile saline solution.

Four weeks after surgery, the animals were euthanized with a lethal injection of anesthetics. The skin was dissected, the calvaria removed, and immediately immersed in a 10% tempered solution of formaldehyde. Micro CT analyses were performed before decalcification.

2.4. Micro-CT

The specimens were scanned using micro-CT (Skyscan 1174; Micro Photonics Inc., Allentown, PA, USA). Before beginning micro-CT scannings, flat-field setups are arranged. Then, scanning was performed with a spatial resolution of 15 μm using 2800 ms 50 kV and 800 μA at a 0.7° rotation steps with 3 frames for a total of 180°. All images were taken in three-dimensional reconstruction with the NRECON software, and then the collected data were evaluated with CTAn software. In that part of analysis, upper and lower borders of the defect are assigned and the region is selected without any healthy tissue contribution in the defect as region of interest between these upper and lower limits. Settings of dark and low gray regions are done for each tissue. After all of these settings 3D analysis performed with only volume of mineralised new bone formation without graft materials was calculated.

2.5. Stereology analysis

The samples were decalcified using formic acid (%5) for 21 days. After the decalcification process, the samples were fixed in 10% formaldehyde, dehydrated in a graded alcohol series, and cleared in xylol for light microscopic examination. After dehydration, specimens were embedded in fresh paraffin. Sections were cut using a microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany). Each paraffin block was serially cut into 7-μm thickness. For volumetric estimation procedure, every 20th section was selected through a set of consecutive paraffin sections from each sample. Choosing the first section was done randomly. All of the sections were sampled from each sample in a systematic random manner. Selected sections were stained with hematoxylin–eosin (H-E) and photographed on the stereology analysis system (Stereo-investigator 9.0, Microbrightfield, Williston, VT, USA) using a light microscope (Leica M 4000 B, Germany) with a digital color camera attachment (Microbrightfield, Williston, VT, USA).

Unbiased Cavalieri method was applied to the light microscopic images for the stereological estimation of volume of new bone area (Vn). Point counting test grids were used for the estimation of these parameters. These grids were used to estimate volume of new bone area (Vn). The point density of the point counting grids was designed to obtain an appropriate coefficient of error (CE) for interesting area in images of the serial sections (Odaci et al., 2003). CE and coefficient of variation (CV) were estimated according to Gundersen and Jensen' formula (Gundersen and Jensen, 1987). The test grid with systematic array of points was randomly placed on the screen of PC. The volume of each interesting area in all sections was estimated with following formula:

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

('t', section thickness; 'a/p', representing area of each point on the point counting grid; '$\sum$p', total number of the points hitting the interesting area).

2.6. Statistically analysis

The Shapiro–Wilk test was used to determine whether the data were normally distributed. Comparisons of the micro-CT and stereological parameters were analyzed using the Kruskal–Wallis nonparametric test, followed by post-hoc group comparisons with the Bonferroni-adjusted Mann–Whitney *U* test after normality of data had been failed. For the Bonferroni correction, $\alpha = 0.05/3 = 0.016$ was considered to be statistically significant. All tests were performed using statistical software (SPSS Inc., version 19.0, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

All animals tolerated surgery well and survived the post-surgical period. Neither wound dehiscence nor wound infection or abscess formation was observed at any surgical site.

3.1. Histological evaluation

Stereologic analysis of new bone formation and connective tissue in all groups were done.

Histological analysis showed new bone formation in all groups. In group C, new bone formation was noted around autogenous bone particles and surrounded by connective tissue. In group RSV-2 and group RSV-5, autogenous grafts were trapped by new bone and in some areas it reaches to defect margins. In both RSV groups connective tissue was superior and irregular between new bone areas (Fig. 1).

3.2. Stereological analysis

The mean bone formation in group RSV-5 and group RSV-2 were 1.49 ± 0.02 mm^3 and 1.41 ± 0.03 mm^3 respectively For both groups, it was a statistically significant difference between group C with a mean bone formation 0.99 ± 0 01 mm^3 ($p \leq 0.05$). Although bone formation in group RSV-5 was more greater than group RSV-2, the difference was not statistically significant (Fig. 2).

Connective tissue volume in group RSV-5 was 1.52 ± 0.03 mm^3 and it was superior than in group RSV-2 (1.47 ± 0.03 mm^3) but the difference was not statistically significant ($p \geq 0.05$). In group C mean connective tissue volume was 0.92 ± 0.02 mm^3 and it was significantly lesser than other groups ($p \leq 0.05$) (Fig. 3).

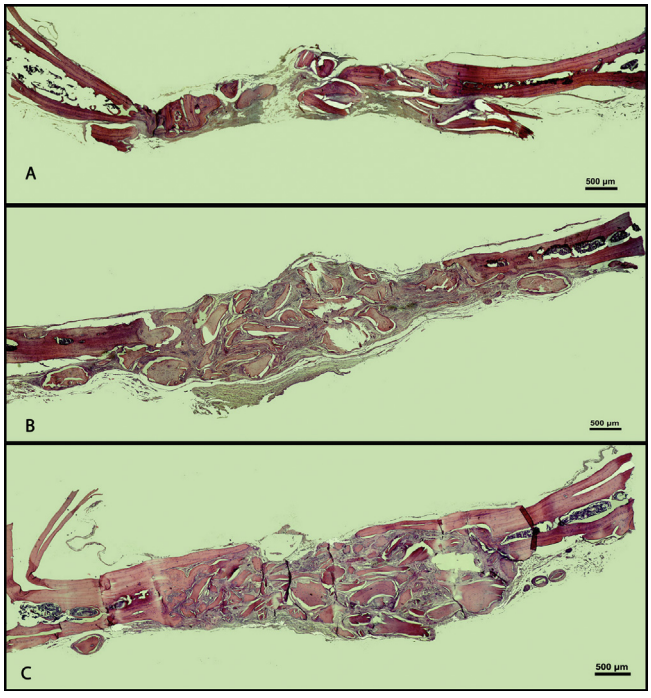

Fig. 1. Panoramic views of the defects. (A) Group C; (B) Group RV-2; (C) Group RSV-5.

3.3. Micro CT analysis

Micro-CT results were shown in Fig. 4. The differences between RSV groups and control group were statistically significant ($p \leq 0.05$). Volume of new bone was higher in RSV-5 than group RSV-2. However, these values were not found statistically significant degree ($p \geq 0.05$). Micro-CT views also were shown in Fig. 5.

4. Discussion

In the present study we hypothesized that rosuvastatin will augment and increase new bone formation with autologous graft in rats. To test this hypothesis we filled 5-mm calvarial critical size defects with autologous graft and administered different doses of rosuvastatin by oral gavage method in rosuvastatin groups. New bone volume was analyzed by micro-CT and stereologic methods.

In the craniomaxillofacial region, extensive large defects cannot heal spontaneously and cause dysfunction and deformity. Clinicians use autologous tissue sources as the gold standard to solve this problem (Zhong et al., 2012). Bone defect models are the preferred approach to investigate the efficiency of biomaterials and stimulatory factors on healing (Petridis et al., 2015). The critical-size rat calvarium defect model was used in this study because it has been used in several investigations in research for the testing of osteopromotive substances. This model provides an inexpensive and easy way to evaluate bone regeneration. In rats, 5-mm calvarial defects are regarded as critical-size defects (Kochi et al., 2009).

Statins are rapidly absorbed after oral administration and their systemic bioavailability is low because of extensive first-pass metabolism in the liver (Schachter, 2004). There are several reports that demonstrated positive effects on bone formation when statins are administered orally (Mundy et al., 1999; Ayukawa et al., 2004). To the best of the authors' knowledge there is no study about effect of oral gavaged RSV on bone defects so we standardized oral doses according to previous simvastatin studies. Karlson et al. (2016) found that each rosuvastatin dose has equivalent effect to doses 7–8 times higher for simvastatin. Also Kaleağasıoğlu et al. (2015) showed positive effect of 20–40 mg simvastatin on bone mineral density. In the light of these studies we decided to evaluate effect of 2–5 mg rosuvastatin.

We choose to use two different methods for analyzing our samples and using stereology and micro-CT analyses made our results more reliable. Histologic examinations are limited to two-dimensional slices, which may fail to identify small islands of bone formation. In 1984, Sterio described several modifications in the approaches used to estimate the quantity of objects in three-dimensional (3D) space. The estimation of microscopic parameters in a 3D space and using micro-CT for 3D imaging increase the reliability of morphological measurements (Novaes et al., 2012; Power et al., 2015).

One of the molecules that have been found to be involved in bone repair are bone morphogenic proteins (BMPs). BMPs are known to play a critical role in transcription of osteoinductive

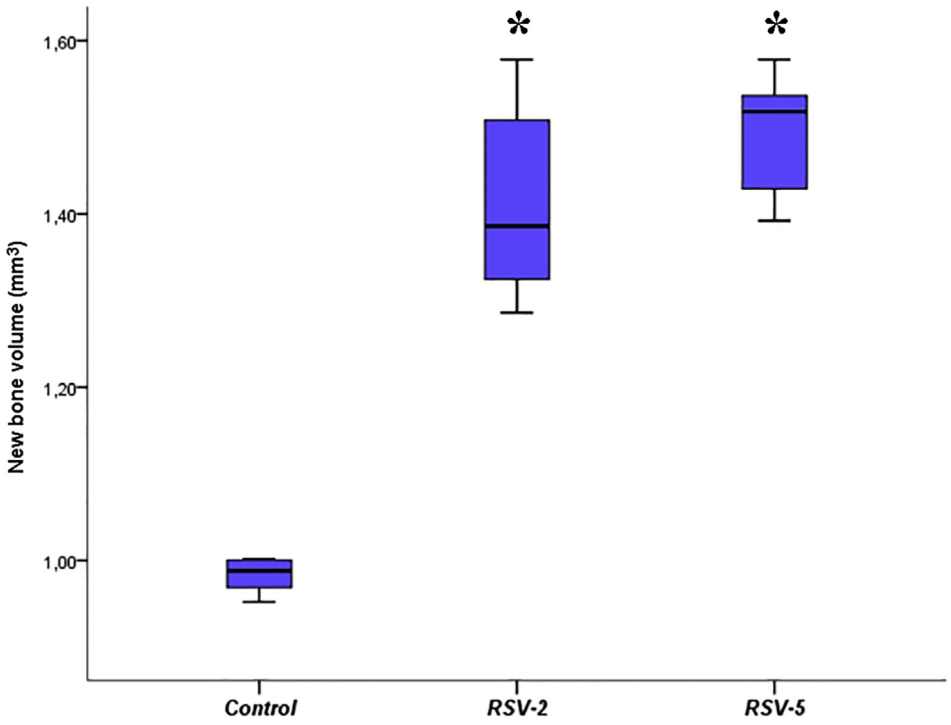

Fig. 2. The new bone volume (mm^3) from stereological analysis in defects. *Statistically significant difference from Control group (Bonferroni-adjusted Mann–Whitney *U* test).

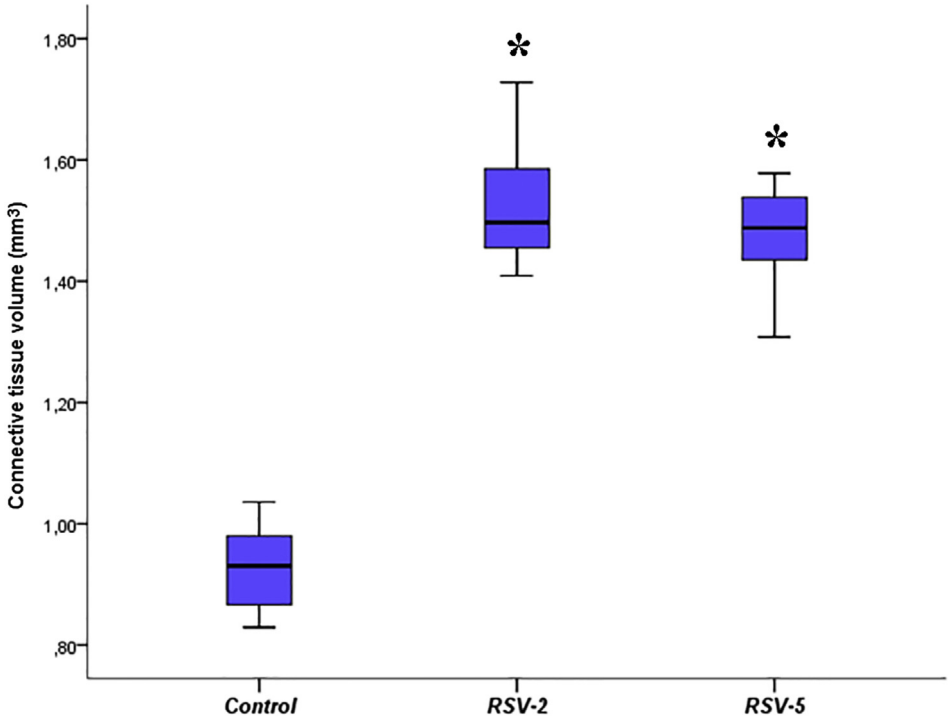

Fig. 3. The connective tissue volume (mm^3) from stereological analysis in defects. *Statistically significant difference from Control group (Bonferroni-adjusted Mann–Whitney *U* test).

genes and enhance osteoblast differentiation (Dimitriou et al., 2005). It is known that statins act on bone by BMP-2 induction and can be responsible for promoting bone growth while inhibiting bone resorption at the same time, as first reported by Mundy et al. (1999).

Some authors reported the positive effects of statins on bone repair. Du et al. (2009) evaluated effects of simvastatin around titanium implants in osteoporotic rat models. They found that simvastatin improves the osseointegration of pure titanium implants in osteoporotic rats. Titanium implants were coated with

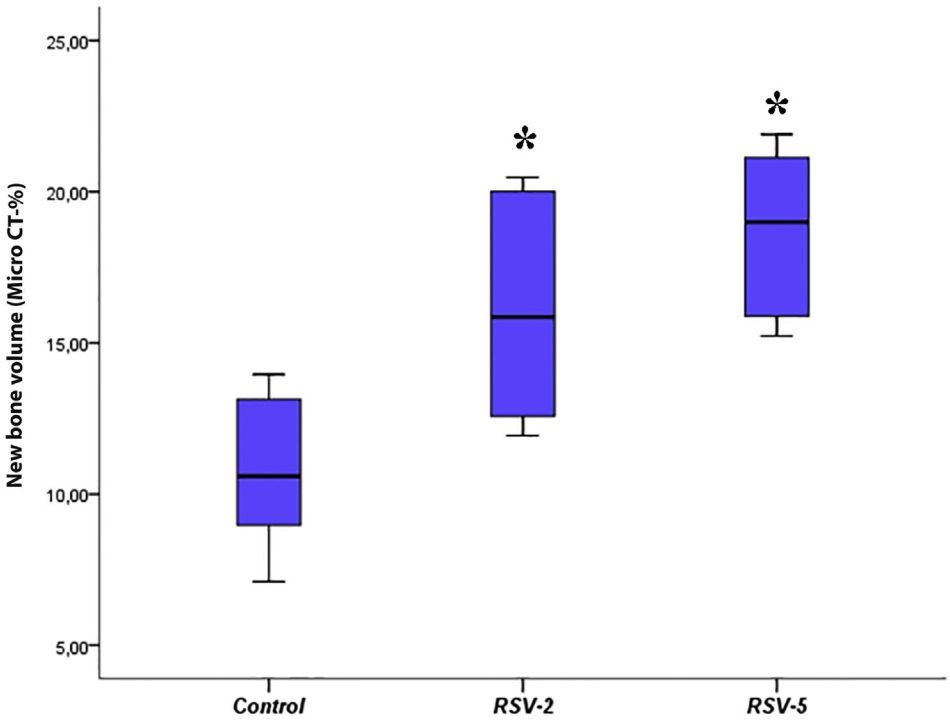

Fig. 4. The percentage of new bone area from the micro-computerized tomography measurements in defects. *Statistically significant difference from Control group (Bonferroni-adjusted Mann–Whitney *U* test).

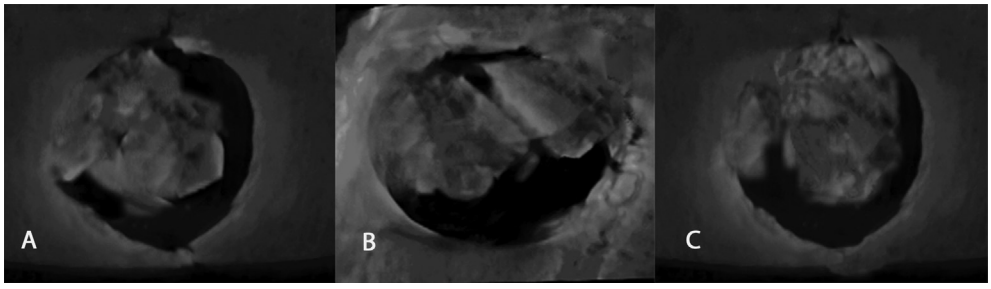

Fig. 5. Micro-CT views of the defects. (A) Group C; (B) Group RV-2; (C) Group RSV-5.

simvastatin and were studied effects of simvastatin on osseointegration in patients with low bone density by Walter et al. (2014). They suggested that surface coating with simvastatin holds potential for use in patients with compromised bone.

RSV, a relatively new HMG-CoA reductase and has the longest terminal half-life compared to the other statins (McTaggart et al., 2001). There are several reports that evaluated different effects of RSV such as; endothelialization of the coiled aneurysm neck (Liu et al., 2015), protecting body mass of HIV infected patients (Erlandson et al., 2016) and biomechanical effect on Achilles tendinopathy (Kaleağasıoğlu et al., 2015). In recent years there have been reports that examine the effects of RSV on bone defects. According to Pradeep et al. RSV has a positive effect on mandibular degree II furcation defects and chronic periodontitis patients. (Pradeep et al., 2015a, b). Monjo et al. (2010) evaluated the potential enhancing effect of RSV on bone formation in critical-size cortical bone defects adjacent to titanium implants. They found that RVS has a potential effect in stimulating bone formation when locally administered. Al-Obaidi et al. (2014) reported that RSV combined with ellagic acid has accelerated the healing process of the tooth socket of diabetic rats after tooth extraction. In our study, we investigated effect of different doses of RSV on critical size calvarial defects in rats.

Stereologic and micro-CT analyses are examined in a three-dimensional space so for these methods error chance is less then conventional histology. Based on stereologic analyses, no difference was found regarding bone formation in the defect area between RSV groups. Consistent with previous statin studies RSV had a positive effect on bone formation compared to control group. (Dimitriou et al., 2005; Al-Obaidi et al., 2014; Pradeep et al., 2015). The values obtained from micro-CT and stereologic evaluations were similar. According to micro-CT results new bone formation is higher in both RSV groups than control group.

Connective tissue volumes were greater in RSV groups according to control group. This situation may be related to inflammation. There are studies about increased inflammation levels with statin treatments. Stein et al. (2005) used different doses of simvastatin and stated that increased simvastatin level shows more inflammation. Calixto et al. (2011) also reported similar results. They showed that animals that received simvastatin presented crust formation, necrosis, and higher levels of inflammation than animals in the control group after 30 days. As a result of inflammatory effect,

RSV may cause an increased amount of connective tissue infiltration since inflammation induces and triggers the migration of connective tissue compounds including cells and intercellular substances. Although it is difficult to say that RSV may increase inflammation since we did not conduct any evaluation which shows inflammation levels, it may be concluded that an excessive amount of connective tissue might be related to inflammatory effect. In our study, when we compared RSV groups, RSV-5 group had higher connective tissue volume but the difference between RSV-2 group was not significant.

5. Conclusion

According to our study results, we conclude that different orally administered doses of rosuvastatin have beneficial effects on healing of experimental defects in the calvaria of rats and may be used with autogenous grafts. Further investigations aimed at finding optimal doses to maximize the anabolic actions of rosuvastatin and the inflammatory effect of systemic RSV on bone is required.

Conflict of interest

None.

Acknowledgments

This research was supported by Scientific Research Project Fund of Bülent Ecevit University with number 2014-68370268-01. Special thanks to research assistants Gamze Yayla and Burcu Delibaş for supporting stereological examination.

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