Effects of exercise training on gingival oxidative stress in obese rats

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List of abbreviations: CRP, C-reactive protein; GSH, reduced-form glutathione; GSSG, oxidized-form glutathione; 8-OHdG, 8-hydroxydeoxyguanosine; ROM, reactive oxidative metabolites; ROS, reactive oxygen species; TRAP, tartrate-resistant acid phosphatase

Abstract

Objective: The purpose of the present study was to investigate the effects of exercise training on serum reactive oxygen species (ROS) level and gingival oxidative stress in obese rats fed a high-fat diet.

Design: Rats were divided into three groups (n=14/group): one control group (fed a regular diet) and two experimental groups (fed a high-fat diet with and without exercise training [treadmill: 5 days/week]). **The rats were sacrificed at 4 or 8 weeks**. The level of serum reactive oxidative metabolites (ROM) was measured as an indicator of circulating ROS. The level of 8-hydroxydeoxyguanosine (8-OHdG) and reduced-form glutathione (GSH)/oxidized-form glutathione (GSSG) ratio were determined to evaluate gingival oxidative stress.

Results: The obese rats fed a high-fat diet **without exercise training** showed higher serum ROM levels **[Carratelli Units (CARR U)] (mean ±SD; 413 ±64)** than the control **(333 ±12)** at 4 weeks ($p = $ **0.023**). Such a condition resulted in higher 8-OHdG levels (ng/mg mtDNA) **(0.97 ±0.18)** ($p < 0.05$) and a lower GSH/GSSG ratio **(17.0 ±3.1)** ($p < 0.05$) in gingival tissues, compared to the control (**0.55 ±0.13** for 8-OHdG and **23.6 ±5.8** for GSH/GSSG ratio) at 8 weeks. In addition, the obese rats fed a high-fat diet with exercise training showed lower serum ROM (**623 ±103**) ($p < $ **0.001**) and gingival

8-OHdG levels (**0.69 ±0.17**) (*p*= **0.012**) than those without exercise training (**1105 ±95**

for ROM and **0.55 ±0.13** for 8-OHdG) at 8 weeks.

Conclusions: Obesity prevention by exercise training may effectively suppress gingival

oxidative stress by decreasing serum ROS in rats.

Introduction

Obesity is a chronic metabolic problem with important public health concerns. There is increasing evidence linking obesity to periodontal disease. Cross-sectional studies have shown a positive relationship between body mass index and periodontal diseases.[1-3] Animal studies also revealed that the gingiva in obese rats exhibited higher densities of polymorphonuclear leukocytes than that in normal rats.[4, 5] Thus, obesity may be a risk factor for periodontal diseases.

Adipose tissue secretes several bioactive substances, including reactive oxygen species (ROS).[6] Studies have shown that obesity is associated with a state of systemic increase in ROS.[7, 8] ROS are products of normal cellular metabolism. However, overproduction of ROS may disturb the tissue oxidative/anti-oxidative balance, **which causes oxidative stress by damaging DNA, lipids and protein.**[9] Recent clinical studies suggested that increased oxidative stress and/or decreased anti-oxidative status are involved in the progression of periodontal diseases.[10-12] In a rat model, gingival oxidative stress was associated with periodontal inflammation[4] and osteoclast differentiation on alveolar bone surface.[13] Therefore, a systemic increase in ROS following obesity may lead to progression of periodontal inflammation and osteoclast differentiation through gingival oxidative stress.

Obesity is also associated with systemic inflammation, which is characterized by increased levels of circulating C-reactive protein (CRP).[14] **A systematic review shows strong evidence that plasma CRP in periodontitis patients is elevated compared with control subjects.[15]** CRP is known to induce oxidative stress.[16, 17] The mechanism by which CRP affects periodontal tissues is unclear; however, elevated blood levels of CRP in obesity may also cause gingival oxidative stress.

Regular exercise offers protection against chronic conditions such as obesity.[18] Protective mechanisms include regulation of body weight and reduction of inflammation in adipose tissue.[19-21] Studies suggest that the reduction of body fat causes a decrease in the formation of oxidants[21-23] and systemic inflammation.[24] Therefore, it is possible that prevention of fat accumulation by exercise training inhibits overproduction of ROS and reduces inflammation in the adipose tissue. Such conditions would **reduce levels of** circulating ROS and systemic inflammation in obesity.

In the present work, we hypothesized that obesity prevention by exercise training might prevent gingival oxidative stress by suppressing the systemic increase of ROS and CRP. 8-hydroxydeoxyguanosine (8-OHdG) is the DNA **fragment. It is** formed when guanine in DNA receives oxidative stress.[25] It is also known that glutathione is a ubiquitous tripeptide that plays a central role in cellular anti-oxidant **defense.**

Oxidized-form glutathione (GSSG) accumulates inside the cells, while reduced-form glutathione (GSH) is decreased by oxidative stress.[26] Therefore, the purpose of this study was to investigate the preventive effects of exercise training on serum ROS level, serum CRP level, gingival 8-OHdG level and gingival GSH/GSSG ratio in obese rats fed a high-fat diet. Serum level of reactive oxidative metabolites (ROM) was measured as an indicator of circulating ROS.[12] The main components of ROM are hydroperoxides (intermediate oxidative products of lipids, peptides and aminoacids), and analysis of these can help to determine the total oxidative status in serum. Furthermore, histopathological evaluation, including inflammatory cell infiltrate and osteoclast differentiation, was performed to confirm whether obesity prevention by exercise training could **prevent periodontal disease.**

1. **Materials** and Methods

2.1. Animals

Forty-two male Wistar rats (8 weeks old) were used in this study. The animals were housed under standard conditions and the room was maintained on a cycle of 12-hour light and 12-hour darkness and at a temperature of 23°C to 25°C. The experimental protocol was approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

2.2. Experimental design

Animals were randomly assigned to either a high-fat (n= 28) or a regular diet protocol (n= 14). The high-fat diet consisted of 32.6% lipid, 43.8% carbohydrate, and 23.5% protein (kcal) (F2HFD1, Oriental Yeast Co., Osaka, Japan).[27] The regular diet (12.5% lipid, 63.2% carbohydrate, and 24.3% protein; kcal) consisted of usual pellet rat chow. Animals fed the high-fat diet were divided into two groups. One group of rats (n= 14) remained sedentary for the 4- or 8-week period (HF-Sed, n= 7 each). The other group of rats (n= 14) was exercise trained for 4 or 8 weeks (HF-Tr, n= 7 each). Animals fed the regular diet remained sedentary for the 4 or 8 weeks (RD-Sed, n= 7 each). Exercise training consisted of continuous running on a motor-driven rodent treadmill (Agawa

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Machine Co., Shimane, Japan) 5 times a week. Exercise intensity was progressively increased from 15 minutes/day at 15 m/minute, 0% slope, up to 60 minutes/day at 26 m/minute, 10% slope, in the last 4 weeks of the program.[28] The condition of the last 4 weeks corresponded to a moderate intensity of ~65% of maximum oxygen consumption.[29]

2.3. Histological analyses

After the animals were sacrificed under general anesthesia, the maxillary molar regions (tooth and periodontal tissues) were resected from each rat and immediately fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. The entire abdominal white adipose tissue (visceral fat) was also dissected and weighed. The tooth and periodontal tissues were decalcified with 10% tetrasodium-ethylenediaminetetraacetic acid aqueous solution (pH 7.4) for 14 days at 4°C. Paraformaldehyde-fixed periodontal and adipose tissue samples were embedded in paraffin and stained with hematoxylin and eosin. In periodontal tissue sections, tartrate-resistant acid phosphatase (TRAP) activity was also detected by the azo dye method to identify osteoclasts **(Fig. 1)**.[13] Histological changes were evaluated using a microscope (BX 51-54, Olympus Co., Tokyo, Japan) by one examiner, who was blinded

to the treatment assignment. In the periodontal tissue, the polymorphonuclear leukocytes per standard area (0.05 mm × 0.1 mm) were counted in the connective tissues subjacent to the junctional epithelium.[4] The distance between the cemento-enamel junction and alveolar bone crest was also measured to evaluate the level of alveolar bone resorption.[4] TRAP-positive osteoclasts occurring along the edge of the alveolar bone surface were counted and reported as number/mm.[13]

2.4. Measurement of the gingival 8-OHdG level

Mitochondrial DNA was isolated from rat gingiva using a DNA extractor kit (Wako Pure Chemical Industries, Osaka, Japan). Level of 8-OHdG in the isolated mitochondrial DNA was analyzed using an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan).[30]

2.5. Measurements of the gingival glutathione level

The levels of total glutathione (GSH + GSSG level) and GSSG were determined with colorimetric assay kits (Dojindo Laboratories, Tokyo, Japan).[30] GSSG was determined after blocking GSH with 2-vinylpyridine and the GSH/GSSG ratio in each sample was calculated.

2.6. Measurements of circulating ROM and CRP

Blood samples were collected after 8 weeks directly from the heart of 24-hour-fasted animals. Blood was allowed to clot at room temperature, and serum was separated by centrifugation at 1,500 $\times g$ for 15 minutes. An *N,N*-diethylparaphenylendiamine ROM test was performed immediately using the free radical elective evaluator (Diacron, Grosseto, Italy) on the same day of blood sampling.[12] This measurement evaluates total hydroperoxides, which are generated by the action of ROS on lipids, peptides and aminoacids and their level reflects the total oxidative burden. The measurement unit was expressed as conventional units (Carratelli Units, where 1 CARR U corresponds to 0.08 mg/dL H_2O_2). In addition, the remaining serum aliquots were stored at -80 °C, and serum levels of C-reactive protein (CRP) were later quantified by a highly sensitive ELISA (Life Diagnostics, Inc., West Chester, UK) after thawing all serum samples.[5]

2.7. Statistical Analysis

Parameters were analyzed statistically using one-way ANOVA followed by Tukey's method **(for comparisons among the RD-Sed, HF-Sed and HF-Tr groups at 4 or 8 weeks)** and *t*-test **(for comparison between 4 and 8 weeks within the RD-Sed,**

HF-Sed or HF-Tr groups). All analyses were performed using a software program (SPSS 15.0 J for Windows, SPSS Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Food consumption, body weight, and adipose tissue weight

There were no significant differences in food consumption among the three groups during the experimental period. Because the foot of a rat in the HF-Tr group was injured during exercise training, the data of this animal were removed from our results.

At baseline, no significant differences in body weight were found among the three groups ($p> 0.05$) (Table 1). The rats in the HF-Sed group had greater body weight ($p< 0.05$), visceral fat pad weight ($p< 0.05$), and subcutaneous fat pad weight ($p< 0.05$) than those in the RD-Sed group at 4 and 8 weeks. The rats in the HF-Tr group also had greater visceral fat pad weight ($p< 0.05$) and subcutaneous fat pad weight ($p< 0.05$) than those in the RD-Sed group at 4 weeks. However, the rats in the HF-Tr group had lower visceral fat pad weight ($p< 0.05$) and subcutaneous fat pad weight ($p< 0.05$) than those in the RD-Sed and HF-Sed groups at 8 weeks. In addition, in the RD-Sed and HF-Sed groups, body weight, visceral fat pad weight and subcutaneous fat pad weight at 8 weeks increased compared to those at 4 weeks ($p< 0.05$). In the HF-Tr group, subcutaneous fat pad weight at 8 weeks decreased compared to that at 4 weeks ($p< 0.05$), while body weight at 8 weeks increased compared to that at 4 weeks ($p< 0.05$).

3.2. Histological evaluation

At 4 weeks, there were no significant differences in polymorphonuclear leukocyte density ($p>0.05$), distance between the cemento-enamel junction and alveolar bone crest ($p>0.05$), and number of TRAP-positive osteoclasts ($p>0.05$) among the three groups (Table 2). At 8 weeks, the polymorphonuclear leukocyte density and number of TRAP-positive osteoclasts in the HF-Sed group were higher than those in the RD-Sed group ($p<0.05$) (Fig. 1). The numbers of polymorphonuclear leukocytes and TRAP-positive osteoclasts in the HF-Tr group were lower than those in the HF-Sed group at 8 weeks ($p<0.05$). No significant differences in the distance between the cemento-enamel junction and alveolar bone crest were observed among the three groups at 8 weeks ($p>0.05$). Furthermore, in the HF-Sed group, distance between the cemento-enamel junction and alveolar bone crest and the number of TRAP-positive osteoclasts at 8 weeks increased compared to those at 4 weeks ($p<0.05$). In the HF-Tr group, the polymorphonuclear leukocytes density at 8 weeks decreased compared to that at 4 weeks ($p<0.05$).

3.3. Gingival 8-OHdG level and GSH/GSSG ratio

At 4 weeks, the gingival 8-OHdG level of the HF-Sed and HF-Tr groups was 115% and

109% higher compared with that of the RD-Sed group ($p < 0.05$) (Table 3). The gingival 8-OHdG level of the HF-Sed group was 76% higher compared with that of the RD-Sed group at 8 weeks ($p < 0.05$). The gingival 8-OHdG level of the HF-Tr group was 29% lower compared with that of the HF-Sed group at 8 weeks ($p < 0.05$). No significant difference in gingival 8-OHdG level between 4 and 8 weeks was found in any group ($p > 0.05$). In addition, the gingival GSH/GSSG ratio of the HF-Sed group was 28% lower compared with that of the RD-Sed group at 8 weeks ($p < 0.05$). This value decreased compared to that at 4 weeks (29%) ($p < 0.05$). There was no significant difference in gingival GSH/GSSG ratio between the RD-Sed and HF-Tr groups at 8 weeks ($p > 0.05$).

3.4. Plasma ROM and CRP levels

At 4 weeks, the serum levels of ROM and CRP in the HF-Sed group were higher than in the RD-Sed group ($p < 0.05$) (Table 4). The serum level of ROM in the HF-Tr group was also higher than in the RD-Sed group at 4 weeks ($p < 0.05$). At 8 weeks, the serum levels of ROM and CRP in the HF-Sed group were higher than in the RD-Sed group ($p < 0.05$). On the other hand, the serum levels of ROM and CRP in the HF-Tr group were lower than in the HF-Sed group at 8 weeks ($p < 0.05$). Furthermore, in all groups, the serum ROM level at 8 weeks increased compared to that at 4 weeks ($p < 0.05$), while there was

no significant difference in serum CRP level between 4 and 8 weeks ($p> 0.05$).

4. Discussion

In the present study, the rats fed a high-fat diet showed greater body weight, fat accumulation and the serum ROM level than those fed the control diet at 4 weeks. Such conditions induced a high 8-OHdG level and a low GSH/GSSG ratio in gingival tissues. The polymorphonuclear leukocyte infiltration and number of TRAP-positive osteoclasts on the alveolar bone surface was greater than the control at 8 weeks. On the other hand, exercise training suppressed the effects of the high-fat diet on gingival 8-OHdG level, polymorphonuclear leukocyte infiltration and the formation of TRAP-positive osteoclasts at 8 weeks, thus suppressing fat accumulation and lowering serum ROM elevation. Exercise training can thus effectively suppress the effects of obesity on gingival oxidative stress and periodontal disease by decreasing circulating ROS.

Previous studies have shown that regular exercise markedly reduced oxidative stress (plasma malondialdehyde level) in obese, type 2 diabetic patients[31] and improved blood level of oxidative stress-defense parameters in overweight and obese type 2 diabetics.[32] These observations are consistent with the present results, and it is feasible that the suppression of obesity by exercise training might contribute to a decrease in circulating ROS. Moreover, in this study, the rats with exercise training showed a gingival 8-OHdG level lower than the group without exercise training at 8 weeks. These

findings indicate that exercise training could improve local alterations in gingival redox balance as well as decrease circulating oxidative stress in obesity.

A clinical study revealed that fat accumulation is closely correlated to the systemic increase of ROS.[33] In the present study, exercise training suppressed fat accumulation and lowered serum ROM level in the rats fed a high-fat diet. These findings indicate that the preventive effects of exercise training on fat accumulation resulted in suppression of serum ROS elevation. On the other hand, although the body weight and adipose tissue weight in the obese rats with exercise training were lower than those in the control rats, there were no significant differences in serum ROM level between these two groups at 8 weeks. Even if exercise training can reduce fat accumulation below the normal level, its suppressive effects on serum ROS level may have a limit.

We found that exercise training did not modulate the effects of a high-fat diet on fat pad weight at 4 weeks. Furthermore, the levels of serum ROM and gingival 8-OHdG did not differ in obese rats with and without exercise training at 4 weeks. It is reported that fat accumulation causes an increase in the formation of oxidants.[21-23] In addition, it is known that the degree of gingival oxidative stress is associated with the blood ROS level.[4] Therefore, because fat accumulation did not change, it is feasible that exercise training had little effect on gingival oxidative stress at 4 weeks. An exercise period

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above a certain level may be necessary to suppress gingival oxidative stress following

obesity.

Recent animal studies have demonstrated that osteoclast differentiation is

stimulated by oxidative stress.[13, 34] This evidence indicates that oxidative stress plays a

crucial role in osteoclast differentiation. In this study, the obese rats with exercise

training exhibited a lower number of TRAP-positive osteoclasts than those without

exercise training at 8 weeks. Reduction of gingival oxidative stress by exercise training

may result in inhibition of osteoclast differentiation induced by obesity. However, the

distance between the cemento-enamel junction and alveolar bone crest did not change

following obesity or exercise training during the experimental period. In our model, at 8

weeks, the degree of gingival oxidative stress induced by obesity might be enough to

induce osteoclast differentiation, but not enough to influence alveolar bone resorption

(e.g., alveolar bone height). On the other hand, it is also possible that alveolar bone

resorption will be observed in a later evaluation. Thus, it may take more periods to

observe alveolar bone resorption by obesity in this model.

At 4 weeks, obesity increased the gingival 8-OHdG level, but it did not affect the

gingival GSH/GSSG ratio. Such conditions did not show any pathological changes in

the periodontal tissue. On the other hand, at 8 weeks, obesity induced both a higher

gingival 8-OHdG level and a lower gingival GSH/GSSG ratio than the control, resulting

in osteoclast differentiation on alveolar bone. The combination of oxidative stress and

anti-oxidant defense in gingival tissues might be responsible for inducing alveolar bone

loss.

Studies have demonstrated that exercise training has an anti-inflammatory effect in

obesity.[35, 36] In the present results, the plasma CRP level in obese rats with exercise

training was lower than in those without exercise training. Therefore, it is possible that

the decreases in not only blood ROS but also low-grade systemic inflammation had

beneficial effects on gingival oxidative stress. In the future, additional assays for

measuring pro-inflammatory cytokines in serum and gingival samples may be required

to investigate whether exercise training induces any antioxidative or anti-inflammatory

effects.

The present study had a few limitations. For instance, the present study did not

include a non-obese group that received exercise training. Therefore, the actual effect of

exercise training on the normal gingiva remains unclear. In addition, we did not

examine alveolar bone density. It is possible that there were significant differences in

alveolar bone density among the three groups, although the distance between the

cemento-enamel junction and alveolar bone crest did not differ in our results. It would

be necessary to investigate how obesity and exercise training affect alveolar bone density in the present rat model.

In conclusion, in the rat model, exercise training could inhibit obesity-induced gingival oxidative stress, periodontal inflammation, and osteoclast differentiation by decreasing the serum ROS and CRP levels.

Acknowledgements

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Table 1. Body Weight and Fat Pad Weight in Response to 4- **and** 8-week Interventions
(g, Mean ± SD)

	RD-Sed (N= 7)	HF-Sed (N= 7)	HF-Tr (N= 6)
Body weight at baseline	319 ± 9	325 ± 8	324 ± 3
Body weight at 4 weeks	326 ± 13	368 ± 25^a	350 ± 11
Body weight at 8 weeks	434 ± 32*	476 ± 17^a*	429 ± 27^b*
Visceral fat at 4 weeks	4.0 ± 0.4	5.5 ± 1.0^a	5.3 ± 1.3^a
Visceral fat at 8 weeks	9.3 ± 1.5*	10.5 ± 1.3^a*	6.3 ± 0.8$^{a, b}$
Subcutaneous fat at 4 weeks	1.6 ± 0.3	2.2 ± 0.4^a	2.0 ± 0.6^a
Subcutaneous fat at 8 weeks	3.3 ± 0.9*	4.5 ± 0.7^a*	1.4 ± 0.4$^{a, b}$*

[a] Significantly different from the RD-Sed group, $P < 0.05$ (Tukey's method).

[b] Significantly different from the HF-Sed group, $P < 0.05$ (Tukey's method).

* Significantly different from the value at 4 weeks (within groups), $P < 0.05$ (*t*-test).

Table 2. Histopathological Evaluation in Periodontal Tissues at 4- **and** 8-week Interventions (Mean ± SD)

	4 Weeks			8 Weeks		
	RD-Sed	HF-Sed	HF-Tr	RD-Sed	HF-Sed	HF-Tr
	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 6)
Polymorphonuclear leukocytes (numbers/ 0.05 mm × 0.1 mm)	0.5 ± 0.4	0.9 ± 0.7	1.0 ± 0.7	0.3 ± 0.2	0.8 ± 0.2[a]	0.2 ± 0.2[b, ∗]
Distance between the cemento-enamel junction and the alveolar bone crest (μm)	569 ± 94	585 ± 80	607 ± 52	574 ± 157	739 ± 128*	663 ± 88
TRAP-positive osteoclasts (numbers/mm)	2.8 ± 1.7	3.0 ± 2.1	3.0 ± 1.7	2.0 ± 1.3	7.1 ± 2.1[a, ∗]	3.0 ± 1.7[b]

[a] Significantly different from the RD-Sed group, $P < 0.05$ (Tukey's method).

[b] Significantly different from the HF-Sed group, $P < 0.05$ (Tukey's method).

∗ Significantly different from the value at 4 weeks (within groups), $P < 0.05$ (*t*-test).

Table 3. Gingival Oxidative Stress at 4- **and** 8-week Interventions (Mean ± SD)

	4 Weeks			8 Weeks		
	RD-Sed	HF-Sed	HF-Tr	RD-Sed	HF-Sed	HF-Tr
	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 6)
8-OHdG level (ng/mg mtDNA)	0.47 ± 0.14	1.01 ± 0.32^a	0.98 ± 0.49^a	0.55 ± 0.12	0.97 ± 0.18^a	0.69 ± 0.17^b
GSH/GSSG ratio	23.2 ± 1.4	24.0 ± 1.8	23.7 ± 1.1	23.6 ± 5.8	$17.0 \pm 3.1^{a,} *$	20.4 ± 3.7

[a] Significantly different from the RD-Sed group, $P < 0.05$ (Tukey's method).

[b] Significantly different from the HF-Sed group, $P < 0.05$ (Tukey's method).

* Significantly different from the value at 4 weeks (within groups), $P < 0.05$ (*t*-test).

Table 4. Serum ROM and CRP Levels at 4- **and** 8-week Interventions (Mean ± SD)

	4 Weeks			8 Weeks		
	RD-Sed	HF-Sed	HF-Tr	RD-Sed	HF-Sed	HF-Tr
	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 7)	(N= 6)
ROM (CARR U)	333 ± 12	413 ± 64[a]	466 ± 60[a]	407 ± 19[*]	1105 ± 95[a, *]	623 ± 103[a, b, *]
CRP (mg/mL)	0.40 ± 0.07	0.52 ± 0.15[a]	0.38 ± 0.10	0.33 ± 0.04	0.48 ± 0.04[a]	0.39 ± 0.02[b]

[a] Significantly different from the RD-Sed group, $P < 0.05$ (Tukey's method).

[b] Significantly different from the HF-Sed group, $P < 0.05$ (Tukey's method).

* Significantly different from the value at 4 weeks (within groups), $P < 0.05$ (*t*-test).

Figure Legend

Figure 1. TRAP-positive osteoclasts in rat periodontal ligament at 8 weeks. There were little positive TRAP-positive osteoclasts (arrow, red-stained cytoplasm) in the RD-Sed group (A). The HF-Sed group (B) showed the number of TRAP-positive osteoclasts more than the RD-Sed (A) and the HF-Tr groups (C). AB, alveolar bone and CM, cementum. Scale bar = 30 μm.

Figure 1
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