

## Beneficial effects of fasting regimens on periodontal tissues in experimental periodontitis mice model

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### Abstract

Previous studies demonstrated that fasting has beneficial effects on overall health, extending lifespan and that it rejuvenates cells, promoting tissue regeneration. Periodontitis is a prevalent oral inflammatory disease that leads to alveolar bone loss and linked to several systemic health problems. Ligature-induced periodontitis model is considered to be similar to human periodontitis in various aspect and is one of the most used animal models in periodontal research. The present study aims to investigate the effect of fasting regimen in experimentally-induced periodontitis in mice. In this study we applied two different kinds of fasting regimens: Intermittent fasting (IF / every other day fasting) and prolonged fasting (PF / 2 days fasting and 5 days free food intake) for 4 weeks. We found that both fasting regimen groups showed less amount of bone loss compared to the nonfasting groups at the ligature-periodontitis site, furthermore also at the contralateral side of the maxillae where the physiological bone loss occurs. PQCT and calcein-labeled histomorphometric analyses showed higher bone regeneration capacity in the fasting groups than in the nonfasting groups. Bone marrow cells from the femurs of the fasting groups produced more mineralized modules than the nonfasting groups. Data from this study showed fasting would be beneficial for resisting bone destruction in periodontitis and for maintaining periodontal bone health, which could be due to the increase of osteoprogenitor cells in bone marrow.

**Research article (J Int Dent Med Res 2018; 11(2): pp. 362-369)**

**Keywords:** Periodontitis, Intermittent Fasting, Prolonged Fasting, Animal Experiment.

**Received date:** 21 May 2018

**Accept date:** 30 May 2018

### Introduction

Fasting has been practiced for millennia, but, only recently, studies have shed light on its role in adaptive cellular responses that reduce oxidative damage and inflammation, optimize energy metabolism, and bolster cellular protection.<sup>1</sup> Fasting has been acknowledged to have beneficial effects on aging, extending lifespan, improves cognitive and brain functions, has a cardioprotective effect, promote hematopoietic-

stem-cells-based regeneration, neurogenesis and reverse immunosuppression.<sup>2-11</sup> Periodontal disease is the most prevalent chronic inflammatory disease in humans, and it is considered the most urgent oral health concern.<sup>12</sup> Epidemiologic studies suggest that there is an association between periodontal disease and coronary heart disease and other systemic health outcomes<sup>13</sup>, making it possible for fasting to have an extension of benefits towards periodontal tissue health. Therefore we hypothesized fasting might exert benefits for periodontal tissue health. Ligature induced periodontitis mice model has been widely accepted as the fastest and reliable model for periodontitis as it stimulates plaque accumulation, inducing inflammation of periodontal tissue and ultimately leading to periodontal destruction.<sup>14,15</sup> In this study, we examined the

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effects of fasting regimens on periodontal tissue in ligature-induced periodontitis in mice.

## Materials and methods

All experiment's protocols were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University (A2017-212A). Thirty six male C57BL/6 mice, 7-weeks old were purchased (Sankyo Labo service Corporation, Tokyo, Japan) and kept inside the animal room with stable room temperature and 12 hours dark/light cycles. Mice were given the chance of adjusting to the new environment inside conventional mice cages with sterilized distilled water and food *ad libitum* for 1 week. At 8 weeks of age, mice were moved and lived singly in metabolic cages (Metabolica, Sugiyama-Gen, Tokyo, Japan) and given 3 days of acclimation period. Body weight of the mice was measured after the acclimation period, followed by weekly measurement.

**Ligature induced Periodontitis** - Mice were anesthetized intraperitoneally with a mixture of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). A sterile 5-0 silk ligature (Matsuda Medical Industry Ltd. Japan) was then placed around the cervical area of the upper left second molar, 3 knots then made in the cervico-palatal area to ensure the ligature remained in its position. The contralateral side was left unligated and considered as the control. Seven days after the ligature placement the ligatures were removed under anesthesia. Four groups, each containing 9 mice per group were planned for this experiment: (1) L-/F- group, where no ligature and fasting regimens were applied, (2) L+/F- group, where ligature was applied (periodontitis model) but with no fasting regimen, (3) L+/IF group, where ligature was applied (periodontitis model) followed by intermittent fasting, and (4) L+/PF group, where ligature was applied (periodontitis model) followed by prolonged fasting.

**Fasting Regimen** - Animals were given a chance to recover from the anesthesia effect after ligature removal procedure, one of the following fasting regimens were applied the next day for 4 weeks. Intermittent fasting regiment (IF) was given in the form of every other day fasting where mice were denied access of food for 24 hours followed by 24 hours of free access to food (15 cycles). Prolonged fasting (PF) was performed by denying mice to access food for 2 consecutive days followed by 5 days of free food access (4

cycles). Food was given or removed at 5 pm which was 2 hours before dark time, whereas water was given *ad libitum*.

Fluorescence labeling for histomorphometry analysis was performed to evaluate bone formation activity during the experiment period. Calcein was subcutaneously injected (20 mg/kg) at 4 weeks and 1 week before sacrifice (n=3). Four weeks after ligature removal, all mice were sacrificed. Maxillae were harvested and fixed in 10% neutralized buffered formalin for 48 hours and then stored in PBS before being analyzed radiologically and histologically. Femurs were also harvested, immediately kept in alpha-MEM (Sigma Aldrich, St. Louis) for cell culture experiment.

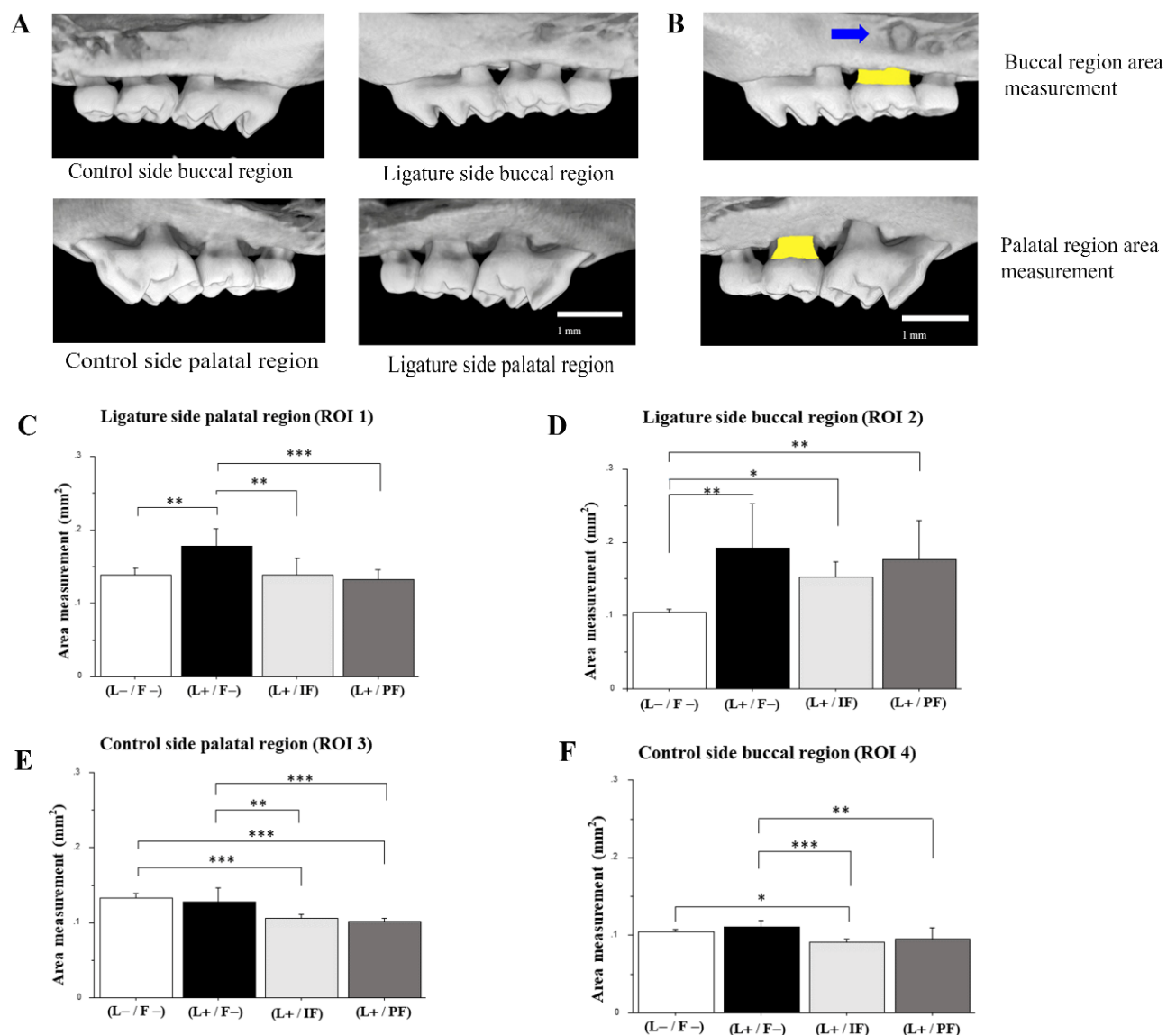
**Micro CT** - To measure the amount of bone loss, three-dimensional images were generated.<sup>16</sup> The maxillae were analyzed with micro-CT (inspeXio SMX-100CT, Shimadzu, Tokyo, Japan) at 60 kV in voltage and 50µA in beam current. Scans were set at 1024 x 1024 pixel size and 0.0012 mm/voxel in voxel size. The results were further processed with a 3D reconstruction software (TRI/3D BON, RATOC system engineering, Tokyo, Japan). At all buccal and palatal regions at both sides, anatomical landmarks were used to reorient and standardize the alignment of the samples. The amount of bone loss was measured with ImageJ program software (National Institute of Health, Bethesda, MD, USA) at both ligature and control side buccally and palatally (**Figure 1A**). Bone loss was determined by measuring area between cemento-enamel junction (CEJ) to alveolar bone crest (ABC) bordered by the mesial and distal side of the exposed root part (**Figure 1B**).<sup>17</sup>

**pQCT** - Peripheral quantitative computed tomography (pQCT; XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany) were used to evaluate bone formation in the form of the amount of cortical area. The maxilla sample was positioned so that the slices would be in a coronal plane manner, a reference line was positioned at the mesial part of upper first molar of the ligature side, with 0.20 mm distance between the reference line and the first slice, continued with 0.30 mm distance between slices. The threshold of 690 mg/cm<sup>3</sup> was used to capture cortical bone (**Figure 2A**), 10 slices were made with the seventh identified as at the upper second molar based on manual width measurement of the molars (**Figure 2B**). The seventh slice was chosen as it allows to differentiate the region of

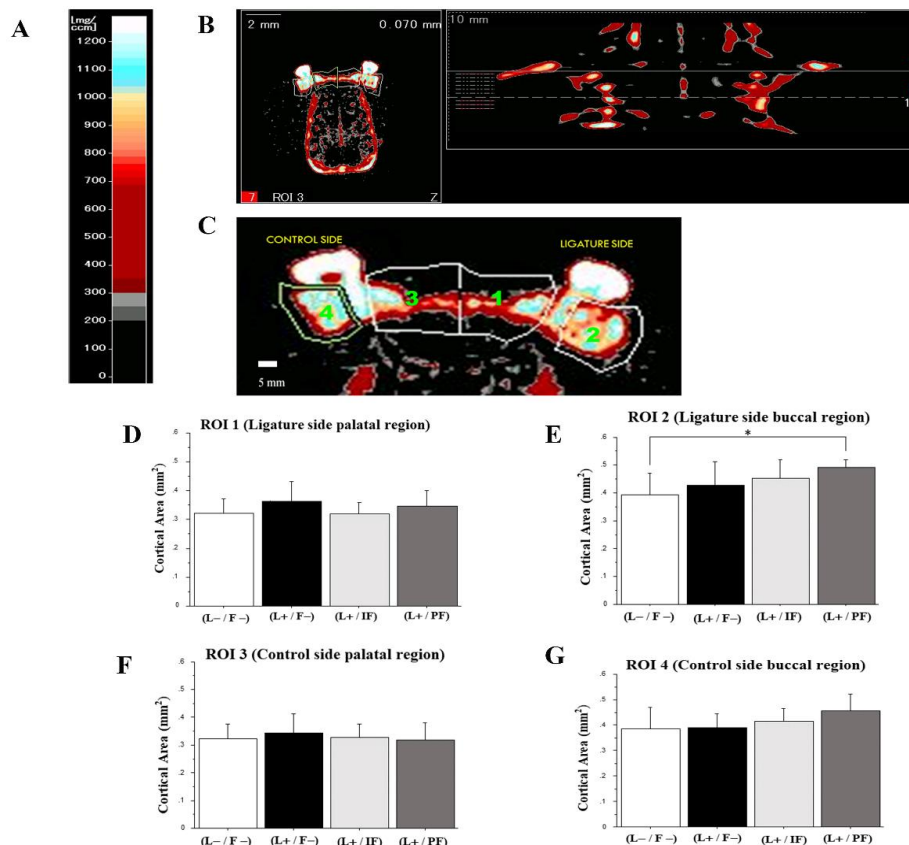
interests (ROI) into; ROI 1 which is the palatal region of ligature side, ROI 2 is the buccal region of ligature side, ROI 3 is the palatal region of control side, and ROI 4 is the buccal region of control side (Figure 2C).

**Histomorphometry** - The maxillae (n=3 / group) were made into frozen samples with a combined protocol of using an embedding material (Super Cryoem bedding medium Compound/SCEM-L1: SECTION-LAB, Hiroshima, Japan) and a freezing machine (UT2000F: Tokyo

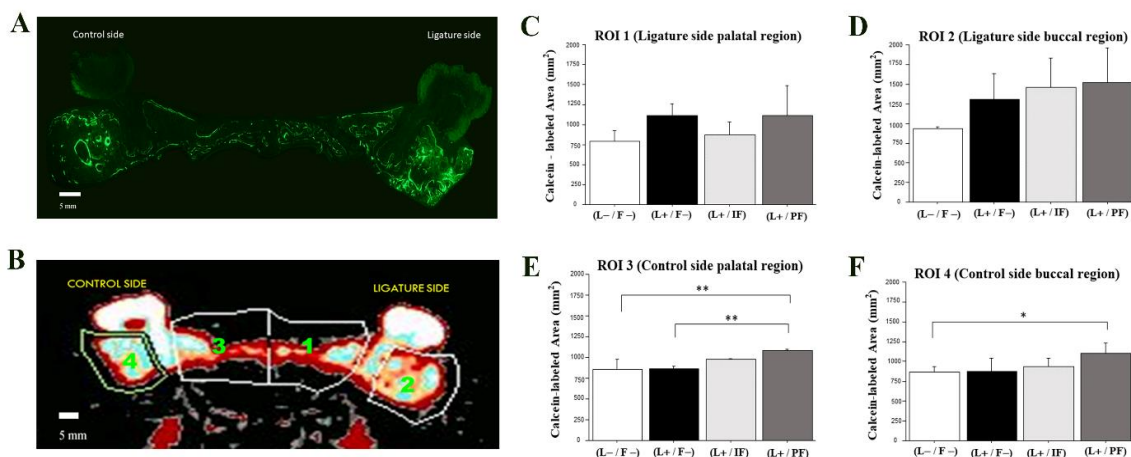
Rikakikai Co. Ltd, Tokyo, Japan). Following Kawamoto's method<sup>18</sup>, an undecalcified 5µm thick sections were prepared with cryomicrotome (CM3050sIV; Leica, Wetzlar, Germany) in a coronal plane manner. Fluorescence images were taken with a microscope (FSX100 Olympus Corporation, Tokyo, Japan) (Figure 3A), the calcein-labeled area was then measured with a software program (image J). The ROIs were determined in the same manner as pQCT analysis (Figure 3B).



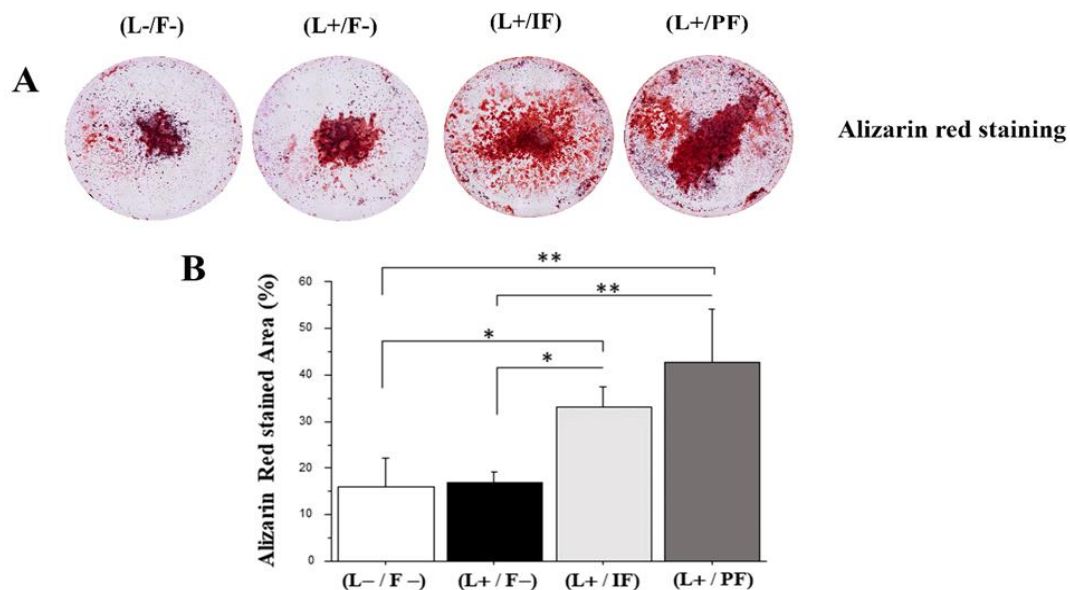
**Figure 1.** A. Representative images of micro ct scan on both ligature and control sides also buccal and palatal regions. B. Bone loss was determined by measuring the area limited by CEJ, ABC, mesial and distal part of the exposed root. Blue arrow indicates fenestration. C. Area measurement at ligature side palatal region (ROI 1) D. Area measurement at the ligature side buccal region (ROI 2). E. Area measurement at control side palatal region (ROI 3). F. Area measurement at the control side buccal region (ROI 4). Mean ± SD (n = 6) was presented, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. L-: no ligature, L+: ligature inserted to induce periodontitis, F-: no fasting, F+: with fasting, IF : intermittent fasting, PF: prolonged fasting.



**Figure 2.** A. Color palette of bone mineral density. B. SV study and slice locating at the area of interest (upper left second molar). C. The region of interest (ROI) mapping of the pQCT slice. D. Cortical area of ROI 1 (ligature side palatal region). E. Cortical area of ROI 2 (ligature side buccal region). F. Cortical area of ROI 3 (control side palatal region). G. Cortical area of ROI 4 (control side buccal region). Mean  $\pm$  SD (n=6) was presented, \* p < 0.05. L-: no ligature, L+: ligature inserted to induce periodontitis, F-: no fasting, F+: with fasting, IF : intermittent fasting, PF: prolonged fasting.



**Figure 3.** A. Fluorescence images are made in pQCT slices manner showing calcein-labeled area indicating bone formation activity. B. pQCT slices orientation and ROI mapping. C. Calcein-labeled area of ROI 1 (ligature side palatal region). D. Calcein-labeled area ROI 2 (ligature side buccal region). E. Calcein-labeled area ROI 3 (control side palatal region). F. Calcein-labeled area ROI 4 (control side buccal region). Mean  $\pm$  SD (n = 3) was presented, \* p < 0.05, \*\* p < 0.01. L-: no ligature, L+: ligature inserted to induce periodontitis, F-: no fasting, F+: with fasting, IF: intermittent fasting, PF: prolonged fasting.



**Figure 4. A.** Alizarin red staining of cell culture wells. **B.** Alizarin red staining area percentage within groups. Mean±SD of three replicates was presented, \* $p < 0.05$ , \*\* $p < 0.01$ . L-: no ligature, L+: ligature inserted to induce periodontitis, F-: no fasting, F+: with fasting, IF: intermittent fasting, PF: prolonged fasting.

**Cell culture experiment** - Bone marrow cells were flushed out from the femurs with a 23 G needle and alpha-MEM, pelleted by centrifugation at 1000g for 5 minutes, the cells pellet then washed with another alpha-MEM and centrifuged one more time similarly. The cells were then cultured under a standard culture condition: alpha-MEM (Sigma-Aldrich, St. Louis) supplemented with 10% FBS and 1 % penicillin/ streptomycin on 10 cm dishes, the medium was refreshed twice a week. Culturing were performed in a humidified 37°C incubator with an atmosphere of 95% air and 5 % CO<sub>2</sub>, the cells from passage two were used for this experiment.

**Alizarin Red Staining** - Cells were seeded into 24 wells plates with a density of 5 x 10<sup>4</sup> per well, cultured in the osteogenic medium supplemented with 10<sup>-8</sup> M dexamethasone, 10 mM β-glycero phosphate (G-9891, Sigma-Aldrich), and 50 ng/mL ascorbic acid (013-12061, Wako Chemical). The culture was stained with alizarin red to detect mineralized nodules at day 21. The cells were washed two times with PBS and fixed with Methanol. After washing with distilled water two times, the culture plate was treated with 1% Alizarin Red S (Sigma-Aldrich A5533) solution (pH 6.4) for 20 minutes followed by two times washing with distilled water. The stained images were captured with a light microscope (Biozero BZ-8000; Keyence, USA) (Figure 4A) and

percentage of the stained area was measured with Image J software (Figure 4B).

**Statistical analysis** - One-way analysis of variance (ANOVA) was used to examine the difference between groups, followed by Fisher's protected least significant difference (PLSD) *post hoc* test. All data were presented as the mean ± SD, and a *p* value of < 0.05 was considered to be statistically significant.

## Results

The weight after acclimation period was considered to be the baseline weight and was used to compare

with the weight at the end of the experiment. Mice body weight was measured once a week. Body weight gain was the highest in the L-/F- group (30%) followed by the L+/F- group (27%), both groups had no limitation to food and water. In the fasting groups, weight gain in the L+/PF group (19%) was high compared to the L+/IF group (1%).

Micro CT analysis showed at the ligature side, the L+/F- group has the highest amount of bone loss, and fasting groups showed significantly less amount of bone loss in the palatal region, but there was no statistically significant difference in the buccal region (Figure 1C, 1D). In the control side at all regions, the fasting groups showed

statistically significant less amount of bone loss compared to the non fasting groups (Figure 1E, 1F).

The effects of fasting regimens to the bone in 4 regions of interest was evaluated by pQCT analysis; there was no statistically significant difference on the total bone mineral density on all regions of interest (data not shown). However cortical area analysis showed the fasting groups had a larger cortical area compared to the non fasting groups in the buccal regions (Figure 2E, 2G).

The calcein-labeled area showed bone formation activity at 4 weeks and 1 week before sacrifice. The total calcein-labeled area was measured to evaluate bone formation activity. At the buccal region of ligature side and all regions of control side, both fasting regimens groups showed higher bone formation activity compared to the nonfasting groups (Figure 3D, 3E, 3F).

Mineralized nodules detected by Alizarin Red S staining showed fasting groups have statistically significant higher percentage area compared to the control groups, with no statistically significant difference between the fasting groups (Figure 4B).

## Discussion

The present study examined the benefits of fasting on ligature-induced periodontitis model. In this study, we applied two different fasting regimens for 4 weeks. The first method is intermittent fasting in the form of every other day fasting or alternate day fasting that involves a "feast day" on which food is consumed ad libitum that alternates with a "fast day" on which food is withheld.<sup>19</sup> The second method is prolonged fasting that was reported by Cheng et al.<sup>3</sup> as fasting period for 48 hours. Body weight is reduced by fasting treatment, especially in the L+/IF group. Studies by Wan et al.<sup>8</sup> found that even though body weight is reduced with intermittent fasting, fasting treatment showed cardioprotective effect. Mice on the fasting regimen groups in this study showed less weight gain compared to nonfasting groups. Nevertheless, both groups showed higher bone formation favorable for periodontal tissue regeneration.

As shown in the micro CT analysis, the amount of bone loss at the ligature side is always highest in L+/F- group, suggesting without any subsequent treatment tissue healing will be

retarded. The fasting groups showed statistically significant less bone loss in the palatal region of ligature side, but at the buccal region, even though there was a tendency of showing a lower amount of bone loss, it was not statistically significant. Similar to Abe & Hajishengallis<sup>20</sup> finding, fenestration (Figure 1B) at the buccal region of ligature side was also seen in several samples, pointing the apical part is thinner than the marginal part. This causes the bone to collapse abruptly, making the buccal side may not be as reliable as the palatal side when performing a morphometric evaluation of bone loss. On the contrary, at the palatal region, the apical part is thicker than the marginal part making bone loss occurs more smoothly (Figure 1C, 1D). The less amount of bone loss in the fasting group at the ligature side implying that fasting improves periodontal tissue healing and regeneration. Previous studies stated distance between CEJ and ABC would increase gradually with age in mice and rats and it is considered to be a physiological bone loss.<sup>21,22</sup> Concordant to Abe & Hajishengallis study<sup>20</sup>, we also found the amount of bone loss on the control side (unligated side) of L+/F- group and L-/F- group had similar value (Figure 1E, 1F). This occurrence led to the belief that the bone loss had nothing to do with inflammation caused by ligature and that it was physiological. The two fasting regimens groups showed less bone loss compared to control groups, implying in the non-ligatured site, fasting prevents the physiological degenerative process. The merits of fasting for periodontal health are further supported in part by pQCT analysis where the fasting groups found to have larger cortical area than the control groups in the buccal region of both sides. Calcein-labeled area analysis further hinted fasting groups tend to have higher bone formation activity compared to L-/F- group, especially in the L+/PF group. These two analyses give additional support to the micro CT results where the two fasting groups showed less bone loss compared to L+/F- group, and less physiological bone loss at the control side (unligated side).

Prolonged fasting is capable of promoting stem cell-based regeneration of the hematopoietic system. A study by Cheng et al.<sup>3</sup> suggests that the effect of prolonged fasting might be a potent strategy to regenerate the hematopoietic and immune systems and possibly other systems and organs. Mesenchymal stem cells (MSCs) are multipotent stem cells that have

the potential to self-renew and differentiate into a variety of specialized cell types such as osteoblasts, chondrocytes, and adipocytes.<sup>23</sup> Bone marrow (BM) is the most common source of MSCs. Two main stem cell population and their progenies, hematopoietic stem cells and BM-MSCs are the main residents of bone marrow.<sup>23,24</sup> We cultured bone marrow stem cells in our study and evaluated mineralized nodules formed under osteogenic induction, the result showed that both fasting methods have higher osteogenic differentiation capacity when compared to the L+/F- group. This is probably the mechanism behind the enhanced condition of periodontal tissue not only at the ligatured site but also at the non-ligatured site. Furthermore, our results suggest that fasting also generates high osteogenic ability of BMMSCs.

Most of earlier studies showed fasting performed prior to injury has the protective effect and help ameliorate the condition resulted by the injuries. A study by Katare, RG et al.<sup>25</sup> found that intermittent fasting applied after induced cardiac injury improves cardiac function and survival from myocardial infarction.

Bao Xin et al.<sup>11</sup> found that prolonged fasting attenuated cerebral ischemic injury, suggesting that fasting not only able to give protective effect but can also be used as a treatment option. The present results imply fasting could be used as a treatment procedure after a disease or injury has situated, mimicking real-life situation where treatment is only applied after the disease has occurred. Further studies are needed to better access the mechanism behind the benefit of fasting on periodontal tissue health.

## Conclusions

The present study shows following ligature removal and subsequent treatment, fasting regimens could enhance periodontal tissue regeneration. This finding is supported with in vitro data whereby cells obtained from groups of animal received fasting treatment have higher osteogenic potential than that of cells obtained from groups of animal without fasting treatment.

## Acknowledgements

We thank Dr. Yukihiko Tamura for his generous support, also Dr. Keo Preksa and Dr. Sun Quan for their technical support in this study.

LKW thanks Dr. Lisa R. Amir, for her valuable advice and support.

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