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Synergic Effects of Mixed Formula Consisted of Polycan and Calcium-gluconate on the Experimental Periodontitis and Alveolar Bone Loss in Rats

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Abstract

Objective : Polycan, exopolymers purified from *Aureobasidium pullulans* SM-2001 and calcium gluconate have been showed favorable inhibitory effects on the periodontitis and related alveolar bone losses through anti-oxidant and anti-inflammatory activities, respectively. In the present study, we intended to observe the possible synergic effects of mixed formula consisted of Polycan and calcium gluconate on ligation-induced experimental periodontitis and related alveolar bone losses in rats, and to select the fittest compositions for further developing as effective agents to ameliorate periodontal diseases.

Method : Experiments were conducted as two separated two tests - first is synergic effects of Polycan and calcium gluconate 1:1, 1:9 and 9:1 mixtures, and second is 1:99, 2:98, 4:96, 8:92 and 1:9 mixtures. Experimental periodontal diseases were induced by ligature placed around the cervix of upper left incisior teeth of rats. One day after ligation placements, 200mg/kg of each single or mixed formulas of Polycan or/and calcium gluconate were orally administered for 10 days. The changes on the alveolar bone loss index and maxillary bone mineral density (BMD) were observed for detecting alveolar bone losses, and for anti-inflammatory effects, myeloperoxidase (MPO) activities and proinflammatory cytokine (tumor necrosis factor; TNF-α) contents were also evaluated in gingival tissues around ligature placed incisior teeth. The results of mixtures were compared with those of singe Polycan and calcium gluconate treated rat.

Results : Each single or mixed formulas of Polycan or/and calcium gluconate favorably and significantly inhibited the inflammatory changes. The inhibitory effects of mixed formula consisted of Polycan and calcium gluconate 1:9 showed against periodontitis and related alveolar bone losses as compared with those of each Polycan and calcium gluconate single formula ($p<0.05$). In second experiment, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 1:9 mixed formulas also showed significant increased anti-inflammatory and inhibitory effects against alveolar bone losses as compared with those of each single formula. Among them, Polycan and calcium gluconate 2:98 showed the highest efficacy against to ligation-induced experimental periodontitis and related alveolar bone losses.

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Conclusion : The results obtained in this study suggest that appropriated mixtures of Polycan and calcium gluconate showed synergic inhibitory effects against ligation-induced experimental periodontitis and related alveolar bone losses in rats. Moreover, Polycan and calcium gluconate 2:98 showed the highest efficacies in this experiment, suggesting the fittest composition for further developing as effective agents to ameliorate periodontal diseases.

Key words : Polycan, Calcium gluconate, Synergic effects, Periodontitis, Alveolar bone loss, Rat

I. Introduction

Periodontitis, a relevant cause of tooth loss in adults[1], is a chronic inflammatory disease that is characterized by localized bone resorption[2),3)]. Recently, involvement of nitric oxide activities, oxidative stresses, in the pathogenesis of periodontitis has been revealed[4], and many antioxidants showed favorable effects on periodontitis and related alveolar bone losses[5),6)]. The induction of periodontal disease by ligature placement is widely used in animal studies, and periodontitis and alveolar bone losses were induced as like humans in this model[7),8)]. Natural products are gaining space and importance in the pharmaceutical industry as well as inspiring the search for new potential sources of bioactive molecules[9]. Herbs, medicinal plants and crude drug substances are considered to be a potential source of antioxidants to combat various diseases including periodontitis and related alveolar bone losses[10].

Polycan is purified exopolymers from *Aureobasidium pullulans* SM-2001, and comprises mostly β-1,3/1,6-glucan and other organic materials, such as amino acids, mono- or di-unsaturated fatty acids (linoleic and linolenic acids), and fibrous polysaccharide[11]. Recently, we found that Polycan has anti-osteoporotic[12),13)]; it inhibited bone losses and accelerated the bone formation, and fracture healing promoting effects[14] with anti-inflammatory effects on xylene-induced acute inflammation[15] and formalin-induced chronic inflammation[16]. It also showed favorable prevention or therapeutic effects on cisplatin-induced kidney damages[17] and on the ligation-induced experimental periodontitis (EPD) and related alveolar bone losses[6] through anti-oxidant and anti-inflammatory mechanisms. Calcium salts (calcium dobestilate, calcium hydroxide, calcium pentosan polysulfate, calcium gluconate) have been showed anti-inflammatory activities[18),19)]. Among them, calcium gluconate have been used for treatment of injuries from direct contact with hydrofluoric acid[20]. It markedly reduced proinflammatory cytokines, interleukin-6 and tumor necrosis factor-α in chemical burns in rats[21]. Indeed, calcium gluconate enhanced anti-inflammatory activities of non-steroid anti-inflammatory drugs[22], and it mitigate the ligation-induced EPD and related alveolar bone losses[5] and collagen-induced rheumatoid arthritis[23] through anti-oxidant and anti-inflammatory mechanisms. It therefore, considered that appropriate mixtures of Polycan and calcium gluconate also might be showed synergic effects on periodontitis and related alveolar bone losses.

In the present study, we intended to observe the possible synergic effects of mixed formula consisted of Polycan and calcium gluconate on ligation-induced EPD and related alveolar bone losses in rats, and to select the fittest compositions for further developing as effective agents to ameliorate periodontal diseases. Changes on the alveolar bone loss index and maxillary bone mineral density (BMD) were observed for detecting alveolar bone losses, and for anti-inflammatory effects, myeloperoxidase (MPO) activities and proinflammatory cytokine (tumor necrosis factor;

TNF-α) contents were also evaluated in gingival tissues around ligature placed incisior teeth in this experiment.

II. Materials and Methods

1. Animals and husbandry

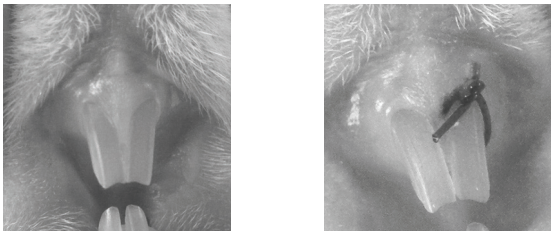
Total one hundred twenty eight healthy male Sprague-Dawley (Slc:SD) rats (6-wk old upon receipt; SLC, Shizuoka, Japan; Body weight ranged in 170~190g), were used after acclimatization for 10 days. Animals were allocated four per polycarbonate cage in a temperature (20-25℃) and humidity (50-55%) controlled room. Light : dark cycle was 12hr : 12hr, and standard rodent chow (Samyang, Korea) and water were supplied free to access. All animals were treated according to the international regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment. Experiments were conducted as two separated two tests - first is synergic effects of Polycan and calcium gluconate 1:1, 1:9 and 9:1 mixtures, and second is 1:99, 2:98, 4:96, 8:92 and 1:9 mixtures. In first experiment, 8 rats per group, total seven groups - intact and EPD control, Polycan and calcium gluconate single formula, Polycan and calcium gluconate 1:1, 1:9 and 9:1 mixed formulas treated groups were selected base on the body weights after 10 days of acclimatization, and also eight rats per group, total 9 groups - intact and EPD control, Polycan and calcium gluconate single formula, Polycan and calcium gluconate 1:99, 2:98, 4:96; 8:92 and 10:90 mixed formulas treated groups were used in second experiment, respectively.

2. Preparations and administration of test materials

Appropriated amounts of Polycan (purified exo-polymers from *Aureobasidium pullulans* SM-2001, contains 14% of β-1,3/1,6-glucan, 18% of β-1,4 -glucan, 8% of α-(1,4)-(1,6)-glucan, 37.7% of glucose, 0.8% of galactose, 1,5% of mannose, 3.1% of protein (3.1%) and 7.2% of ashin dried materials), and calcium gluconate (CAS No. 299-28-5; purity: 98%) were kindly supplied by Aribio Inc. (Seoul, Korea) as brownish and off white powders, respectively. Both materials well dissolved in distilled water, up to 40mg/ml concentration, at least. Appropriate amounts of Polycan or calcium gluconate were dissolved in distilled water as vehicle, and to prepare administration solutions of the mixed formulas consisted of Polycan and calcium gluconate, appropriated amounts of Polycan and calcium gluconate were directly dissolved into distilled water, respectively. One day after ligation placements, 200mg/kg of each single or mixed formulas of Polycan and calcium gluconate were orally administered, once a day for 10 days, in a volume of 5ml/kg, respectively. In intact and EPD controls, same volume of distilled water was orally administered, instead of test substances, once a day for 10 continuous days from 24hrs after ligation placement.

3. Induction of EPD

EPD were induced by sterilized nylon (3-0) thread ligature placed around the cervix of upper left incisior teeth of rats, under anesthetized with 25mg/kg intraperitoneal injection of Zoletile mixture (Zoletile 50; Virbac Lab., Paris, France) according to previous methods[5),6)] with some modifications.



Intact Ligation

Figure 1. Representative images of experimental periodontal disease inducement

The ligature was knotted on the buccal side of the tooth, resulting in subgingival position palatinally and in supragingival position buccally. In intact vehicle control rats, only cervix of upper left incisior teeth was identified, instead of ligation placement in this experiment (Fig 1).

4. Measurements of alveolar bone loss

The animals were sacrificed at 10 days after administration (on 11 days after ligature placement), and maxillary bone contain ligature placement site (second molar) were excised and then, the horizontal alveolar bone loss, the distance between the cusp tip and the alveolar bone, was measured using a modification of the methods of Crawford et al[24] as described by Samejima et al[3]. Measurements were made along the axis of root of the upper left incisior teeth, as mm/rats[25),26)].

5. Measurements of maxillary BMD

At Sacrifice, the upper incisor teeth located maxillary regions were separated from surrounding connective tissues, muscles and any debris, and then dried at 120℃ for 8hrs in high temperature dry oven (LDO-080N, Daihan Labtech Co., Korea). BMD of upper incisor teeth located maxillary regions (5×5mm) was measured by dual-energy x-ray absorptionmetry (Norland pDEXA; Fort Atkinson, WI, USA) after end of 10 days continuous oral administration of test substances or vehicle.

6. Measurement of MPO activity

Gingival tissues around ligature placement were collected at 11 days after EPD induction to determine MPO activity as a measurement of neutrophil accumulation. A spectrophotometric assay was utilized to measure MPO activity, as described previously[27]. The buccal gingival tissues surrounding the left incisior teeth were removed and stored at −70℃. The material was suspended in 0.5% hexadecyltrimethyl-ammonium bromide (Gibco, Carlsbad, CA, USA) in 50mM potassium phosphate buffer, pH 6.0, to solubilize MPO. After homogenized in an ice bath (15s), the samples were freeze-thawed twice. Additional buffer was added to the test tube to reach 400μl of buffer per 15 mg of tissue for 12 min. After centrifuging at 1000×g for 12 min, 0.1 ml of the supernatant was added to 2 ml phosphate buffer (50 mM, pH 6.0), containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma-Aldrich, St. Louise, MO, USA), distillated water and 0.0005% hydrogen peroxide to give a final volume of 2.1 ml per tube. The absorbance was measured by spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea) at 460 nm. One unit of activity was defined as that degrading 1 μmol of peroxide/min at 25℃. Results are expressed as MPO units/ml.

7. Detection of TNF-α contents in rat maxillary gingival tissue

The buccal gingival tissue from the area surrounding the ligature placement was collected at 11 days after EPD induction. The tissue collected was homogenized and processed as described by Safieh-Garabedian et al[28] as described by Botelho et al[26]. The detection of TNF-α concentrations was determined by enzyme linked immunosorbent assay as described previously by Cunha et al[29]. Micro titer plates were coated overnight at 4℃ with antibody against rat TNF-α (10 μg/ml). After blocking the plates, the samples and standard at various dilutions were added in duplicate and incubated at 4℃ for 24 hrs. The plates were washed three times with buffer. After washing the plates, 100 ml of biotinylated sheep polyclonal anti-rat TNF-α (diluted 1/1000 with assay buffer 1% BSA; Abcam, Cambridge, UK), was added to the wells. After further incubation at room temperature for 1 hr, the plates were washed and 100 μl of avidin-HRP (Abcam, Cambridge, UK) diluted 1:5000 were added. The color reagent o-phenylenediamine (100 μl; Sigma-Aldrich, St. Louise, MO, USA) was added 15 min later and the plates were incubated in the dark at 37 ℃ for 20 min. The enzyme reaction was stopped with H_2SO_4 and absorbance was measured using a microplate reader (Tecan, Männedorf, Switzerland) at 490 nm.

8. Statistical analyses

All data were expressed as mean ± standard deviation (SD) of eight rats. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test[30]. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by least-significant differences multi-comparison (LSD) test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity was observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When a significant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, IBM SPSS Inc., Armonk, NY, USA)[31], and p-values < 0.05 were considered significantly different.

III. Results

1. Changes on the alveolar bone loss scores

Significant ($p<0.01$) increases of exposured teeth roots areas, the alveolar bone loss scores were detected in EPD control as compared with intact control in the both first (2.06 mm) and second (2.06 mm) experiment. However, significant ($p<0.01$) decreases of alveolar bone loss scores were detected in all Polycan (−0.49 mm in first, −0.50 mm in second) and calcium gluconate (−0.77 mm in first, −0.75 mm in second) single or mixed formula (−0.63 mm in 1:1, −1.03 mm in 1:9, −0.45 mm in 9:1, −0.82 mm in 1:99, −1.25 mm in 2:98, −1.09 mm in 4:96, −1.06 mm in 8:92, −1.01 mm in 10:90 mixed formula) treated rats as compared with EPD control, respectively. Especially, Polycan and calcium gluconate 1:9 mixed formula treated rats showed significant ($p<0.01$ or $p<0.05$) decreases of bone loss scores as compared with each Polycan (−0.54 mm) and calcium gluconate (−0.26 mm) single formula treated rats in first experiment, respectively. Moreover, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90

mixed formula treated rats also showed significant ($p<0.01$) decreases of bone loss scores as compared with each Polycan (−0.74 mm in 2:98, −0.58 mm in 4:96, −0.55 mm in 8:92, −0.51 mm in 10:90 mixed formula) and calcium gluconate (−0.50 mm in 2:98, −0.34 mm in 4:96, −0.30 mm in 8:92, −0.26 mm in 10:90 mixed formula) single formula treated rats in second experiment, in that orders, respectively(Fig 2).

2. Effects on the maxillary BMD

Significant ($p<0.01$) decreases of maxillary BMD around ligation placed were detected in EPD control

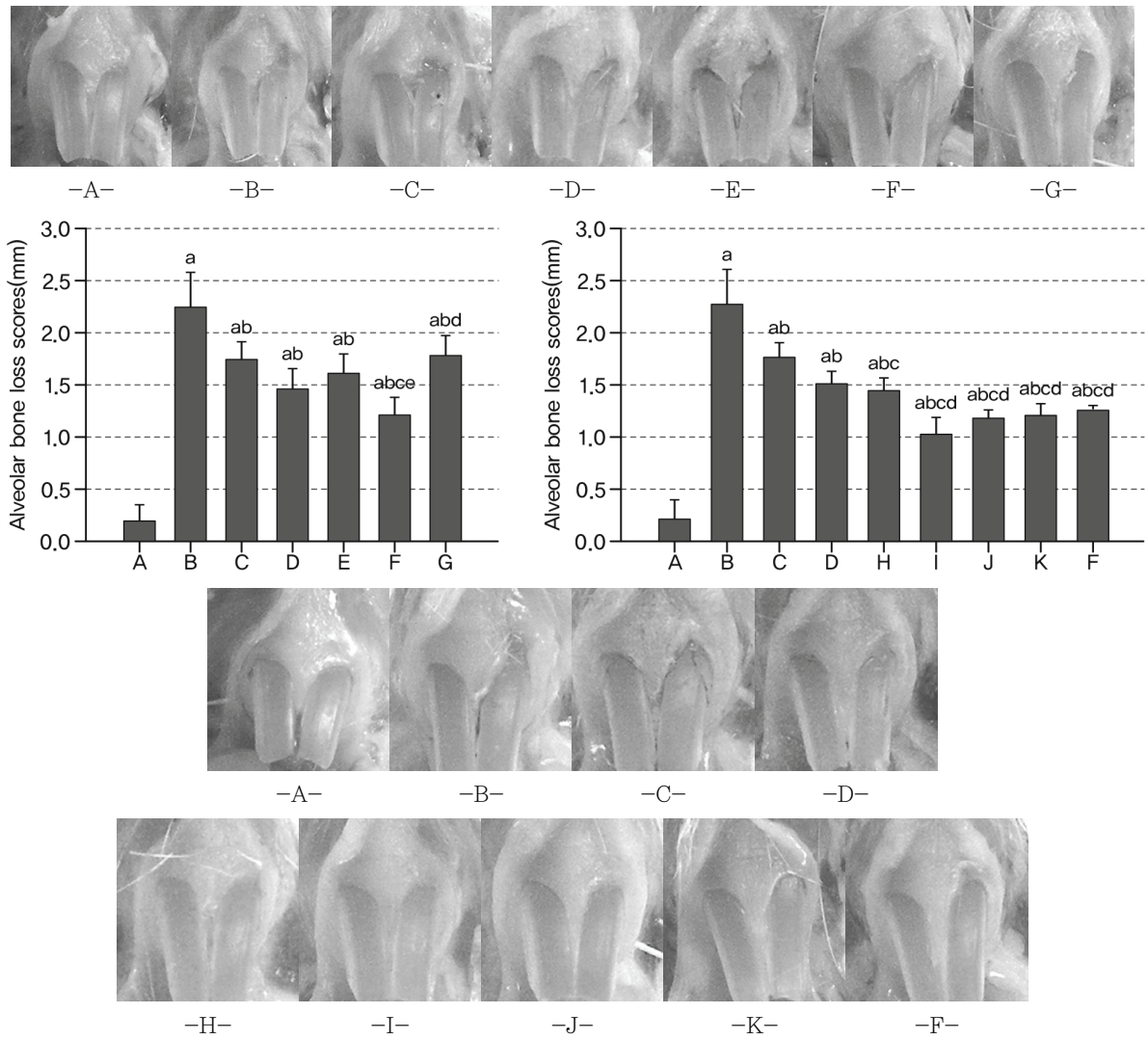


Figure 2. Representative gross images of ligation placed regions and changes on the alveolar bone loss scores after 10 days oral treatment of single or mixed formulas of Polycan and calcium gluconate in EPD rats

A: Intact control, B: EPD control, C: Polycan single formula treated rats, D: Calcium gluconate single formula treated rats, E: Polycan and calcium gluconate 1:1 mixture treated rats, F: Polycan and calcium gluconate 1:9 mixture treated rats, G: Polycan and calcium gluconate 9:1 mixture treated rats, H: Polycan and calcium gluconate 1:99 mixture treated rats, I: Polycan and calcium gluconate 2:98 mixture treated rats, J: Polycan and calcium gluconate 4:96 mixture treated rats, K: Polycan and calcium gluconate 8:92 mixture treated rats.
[a]$p<0.01$ as compared with intact control by LSD test, [b]$p<0.01$ as compared with EPD control by LSD test, [c]$p<0.01$ as compared with Polycan single formula treated rats by LSD test, [d]$p<0.01$ and [e]$p<0.05$ as compared with calcium gluconate single formula treated rats by LSD test

as compared with intact control in the both first (−0.031 g/cm^2) and second (−0.033 g/cm^2) experiment. However, significant ($p<0.01$ or $p<0.05$) increases of maxillary BMD were detected in all Polycan (0.006 g/cm^2 in first, 0.007 g/cm^2 in second) and calcium gluconate (0.010 g/cm^2 in first, 0.011 g/cm^2 in second) single or mixed formula (0.007 g/cm^2 in 1:1, 0.014 g/cm^2 in 1:9, 0.007 g/cm2 in 9:1, 0.011 g/cm^2 in 1:99, 0.021 g/cm^2 in 2:98, 0.019 g/cm^2 in 4:96, 0.016 g/cm^2 in 8:92, 0.014 g/cm^2 in 10:90 mixed formula) treated rats as compared with EPD control, respectively. Especially, Polycan and calcium gluconate 1:9 mixed formula treated rats showed significant ($p<0.01$) increases of maxillary BMD as compared with each Polycan (0.008 g/cm^2) and calcium gluconate (0.004 g/cm^2) single formula treated rats in first experiment, respectively. Moreover, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats also showed significant ($p<0.01$) increases of maxillary BMD as compared with each Polycan (0.014 g/cm^2 in 2:98, 0.013 g/cm^2 in 4:96, 0.010 g/cm^2 in 8:92, 0.008 g/cm^2 in 10:90 mixed formula) and calcium gluconate (0.010 g/cm^2 in 2:98, 0.009 g/cm^2 in 4:96, 0.006 g/cm^2 in 8:92, 0.003 g/cm^2 in 10:90 mixed formula) single formula treated rats in second experiment, in that orders, respectively(Fig 3).

3. Changes on the gingival MPO activities

Significant ($p<0.01$) increases of MPO activities around ligation placed gingival tissues were detected in EPD control as compared with intact control in the both first (74.68 U/mg) and second (76.92 U/mg) experiment. However, significant ($p<0.01$) decreases of gingival MPO activities were detected in all Polycan (−43.12 U/mg in first, −39.55 U/mg in second) and calcium gluconate (−31.85 U/mg in first, −27.84 U/mg in second) single or mixed

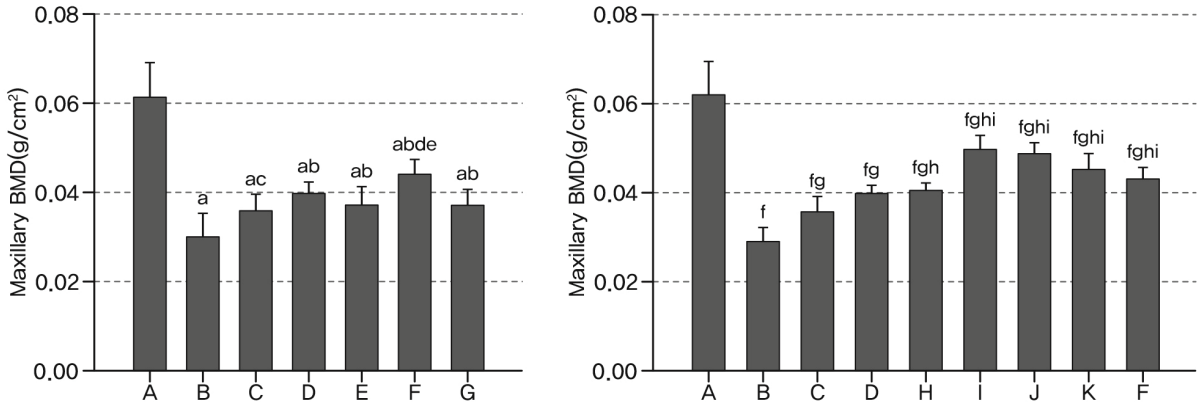


Figure 3. Changes on the maxillary BMD after 10 days oral treatment of single or mixed formulas of Polycan and calcium gluconate in EPD rats

A: Intact control, B: EPD control, C: Polycan single formula treated rats, D: Calcium gluconate single formula treated rats, E: Polycan and calcium gluconate 1:1 mixture treated rats, F: Polycan and calcium gluconate 1:9 mixture treated rats, G: Polycan and calcium gluconate 9:1 mixture treated rats, H: Polycan and calcium gluconate 1:99 mixture treated rats, I: Polycan and calcium gluconate 2:98 mixture treated rats, J: Polycan and calcium gluconate 4:96 mixture treated rats, K: Polycan and calcium gluconate 8:92 mixture treated rats.
[a]$p<0.01$ as compared with in tact control by LSD test, [b]$p<0.01$ and [c]$p<0.05$ as compared with EPD control by LSD test, [d]$p<0.01$ as compared with Polycan single formula treated rats by LSD test, [e]$p<0.01$ as compared with calcium gluconate single formula treated rats by LSD test, [f]$p<0.01$ as compared with intact control by MW test, [g]$p<0.01$ as compared with EPD control by MW test, [h]$p<0.01$ as compared with Polycan single formula treated rats by MW test, [i]$p<0.01$ as compared with calcium gluconate single formula treated rats by MW test

formula (−34.92 U/mg in 1:1, −51.27 U/mg in 1:9, −33.46 U/mg in 9:1, −31.46 U/mg in 1:99, −69.96 U/mg in 2:98, −60.84 U/mg in 4:96, −56.70 U/mg in 8:92, −51.11 U/mg in 10:90 mixed formula) treated rats as compared with EPD control, respectively. Especially, Polycan and calcium gluconate 1:9 mixed formula treated rats showed significant ($p<0.01$) decreases of gingival MPO activities as compared with each Polycan (−9.14 U/mg) and calcium gluconate (−19.42 U/mg) single formula treated rats in first experiment, respectively. Moreover, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats also showed significant ($p<0.01$ or $p<0.05$) decreases of gingival MPO activities as compared with each Polycan (−30.41 U/mg in 2:98, −21.29 U/mg in 4:96, −17.15 U/mg in 8:92, −11.56 U/mg in 10:90 mixed formula) and calcium gluconate (−42.12 U/mg in 2:98, −33.01 U/mg in 4:96, −28.86 U/mg in 8:92, −23.27 U/mg in 10:90 mixed formula) single formula treated rats in second experiment, in that orders, respectively(Fig 4).

4. Effects on the gingival TNF-α contents

Significant ($p<0.01$) increases of TNF-α contents around ligation placed gingival tissues were detected in EPD control as compared with intact control in the both first (655.75 ng/ml) and second (660.25 ng/ml) experiment. However, significant ($p<0.01$) decreases of gingival TNF-α contents were detected in all Polycan (−449.38 ng/ml in first, −452.00 ng/ml in second) and calcium gluconate (−350.75 ng/ml in first, −346.00 ng/ml in second) single or mixed formula (−391.88 ng/ml in 1:1, −534.13 ng/ml in 1:9, −414.50 ng/ml in 9:1, −379.75 ng/ml in 1:99, −601.00 ng/ml in 2:98, −562.25 ng/ml in 4:96, −533.63 ng/ml in 8:92, −512.00 ng/ml in 10:90 mixed formula) treated rats as compared with EPD control, respectively. Especially, Polycan and calcium gluconate 1:9 mixed formula treated rats showed significant ($p<0.01$ or $p<0.05$) decreases of gingival TNF-α contents as compared with each Polycan (−84.75 ng/ml) and calcium gluconate

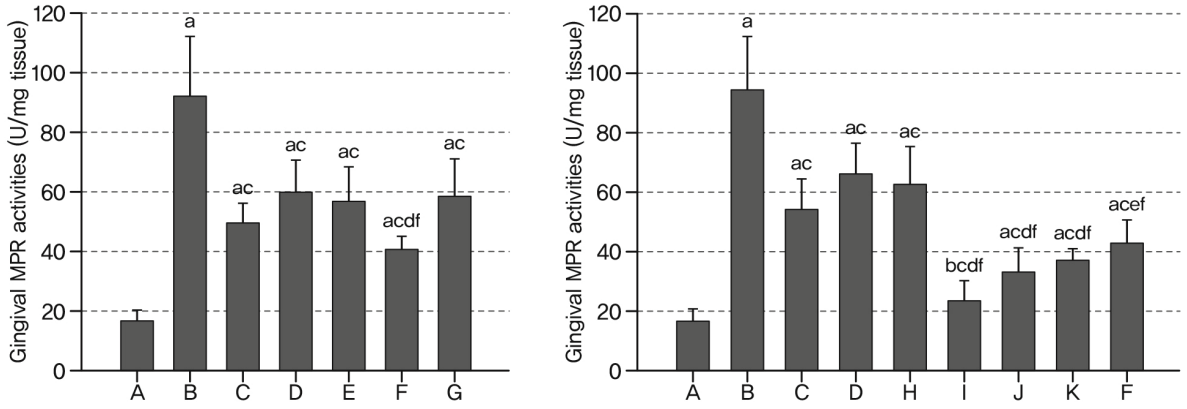


Figure 4. Changes on the gingival MPO activities after 10 days oral treatment of single or mixed formulas of Polycan and calcium gluconate in EPD rats

A: Intact control, B: EPD control, C: Polycan single formula treated rats, D: Calcium gluconate single formula treated rats, E: Polycan and calcium gluconate 1:1 mixture treated rats, F: Polycan and calcium gluconate 1:9 mixture treated rats, G: Polycan and calcium gluconate 9:1 mixture treated rats, H: Polycan and calcium gluconate 1:99 mixture treated rats, I: Polycan and calcium gluconate 2:98 mixture treated rats, J: Polycan and calcium gluconate 4:96 mixture treated rats, K: Polycan and calcium gluconate 8:92 mixture treated rats.
$^{a}p<0.01$ and $^{b}p<0.05$ as compared with intact control by MW test, $^{c}p<0.01$ as compared with EPD control by MW test, $^{d}p<0.01$ and $^{e}p<0.05$ as compared with Polycan single formula treated rats by MW test, $^{f}p<0.01$ as compared with calcium gluconate single formula treated rats by MW test

(−183.38 ng/ml) single formula treated rats in first experiment, respectively. Moreover, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats also showed significant ($p<0.01$ or $p<0.05$) decreases of gingival TNF-α contents as compared with each Polycan (−149.00 ng/ml in 2:98, −110.25 ng/ml in 4:96, −81.63 ng/ml in 8:92, −60.00 ng/ml in 10:90 mixed formula) and calcium gluconate (−255.00 ng/ml in 2:98, −216.25 ng/ml in 4:96, −187.63 ng/ml in 8:92, −166.00 ng/ml in 10:90 mixed formula) single formula treated rats in second experiment, in that orders, respectively(Fig 5).

IV. Discussion

Periodontal disease are representative chronic inflammatory disease, generally periodontitis and related alveolar bone losses were accompanied[2),3)]. We previously revealed that Polycan and calcium gluconate single formula has favorable inhibitory effects on periodontitis and related alveolar bone losses through anti-oxidant and anti-inflammatory activities, respectively[5),6)]. In the present study, we intended to observe the possible synergic effects of mixed formula consisted of Polycan and calcium gluconate on ligation-induced EPD and related alveolar bone losses in rats, and to select the fittest compositions for further developing as effective agents to ameliorate periodontal diseases. Experiments were conducted as two separated two tests - first is synergic effects of Polycan and calcium gluconate 1:1, 1:9 and 9:1 mixtures, and second is 1:99, 2:98, 4:96, 8:92 and 1:9 mixtures.

As results of first and second experiment, each single or mixed formulas of Polycan and calcium gluconate favorably and significantly inhibited the inflammatory changes, the elevation of gin-

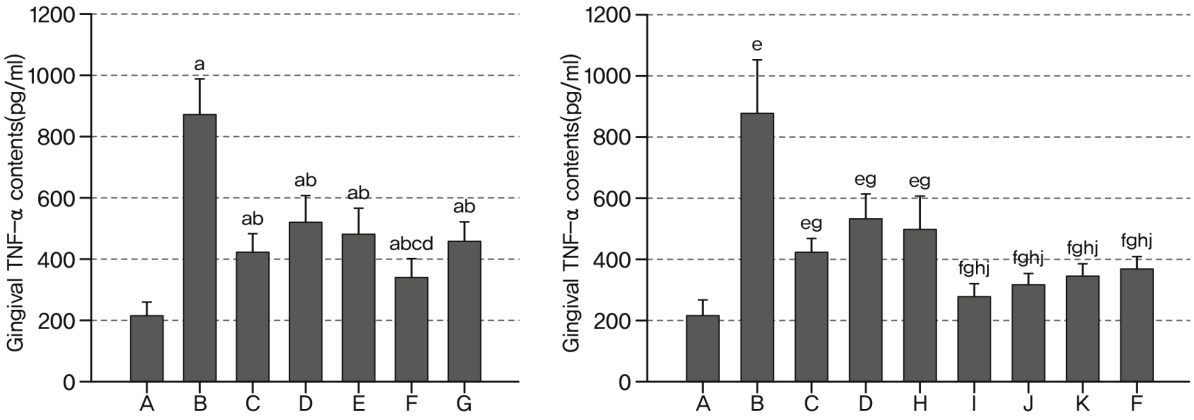


Figure 5. Changes on the gingival TNF-α contents after 10 Days oral treatment of single or mixed formulas of Polycan and calcium gluconate in EPD rats

A: Intact control, B: EPD control, C: Polycan single formula treated rats, D: Calcium gluconate single formula treated rats, E: Polycan and calcium gluconate 1:1 mixture treated rats, F: Polycan and calcium gluconate 1:9 mixture treated rats, G: Polycan and calcium gluconate 9:1 mixture treated rats, H: Polycan and calcium gluconate 1:99 mixture treated rats, I: Polycan and calcium gluconate 2:98 mixture treated rats, J: Polycan and calcium gluconate 4:96 mixture treated rats, K: Polycan and calcium gluconate 8:92 mixture treated rats.
[a]$p<0.01$ as compared with intact control by LSD test, [b]$p<0.01$ as compared with EPD control by LSD test, [c]$p<0.05$ as compared with Polycan single formula treated rats by LSD test, [d]$p<0.01$ as compared with calcium gluconate single formula treated rats by LSD test, [e]$p<0.01$ and [f]$p<0.05$ as compared with intact control by MW test, [g]$p<0.01$ as compared with EPD control by MW test, [h]$p<0.01$ and [i]$p<0.05$ as compared with Polycan single formula treated rats by MW test, [j]$p<0.01$ as compared with calcium gluconate single formula treated rats by MW test

gival tissue MPO and TNF-α contents, and also significantly inhibited the increases of alveolar bone losses, the increases of alveolar bone loss index and decreases of maxillary BMD induced by EPD in the both first and second experiments. Moreover, mixed formula consisted of Polycan and calcium gluconate 1:9 showed significantly increased inhibitory effects against periodontitis and related alveolar bone losses as compared with those of each Polycan and calcium gluconate single formula, but not in 1:1 and 9:1 mixed formulas. In second experiment, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 1:9 mixed formulas also showed significant increased anti-inflammatory and inhibitory effects against alveolar bone losses as compared with those of each Polycan and calcium gluconate single formula, but not in 1:99 mixed formula. Among them, Polycan and calcium gluconate 2:98 showed the highest efficacy against to EPD. These findings are considered as direct evidenced that appropriated mixtures of Polycan and calcium gluconate showed synergic inhibitory effects against ligation-induced EPD and related alveolar bone losses in rats, and Polycan and calcium gluconate 2:98 was the fittest composition for further developing as effective agents to ameliorate periodontal diseases.

The alveolar bone loss scoring system based of exposure of teeth roots from alveolar sockets, is one of generally used macroscopical evaluation to detected alveolar bone losses. In this system, the higher the score, the higher the level of alveolar bone[26),32)]. We found that a significant decrease of alveolar bone loss scores by treatment of all single or mixed formula consisted of Polycan and calcium gluconate, respectively. Especially, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats showed significant decreases of bone loss scores as compared with each Polycan and calcium gluconate single formula treated rats, in that orders, but not in 1:1 and 9:1 mixtures. These results are considered as one of direct evidences that appropriated mixed formulation of Polycan and calcium gluconate showed synergic inhibitory effects against ligation-induced alveolar bone losses, and the fittest composition for further developing as effective agents to ameliorate periodontal diseases is Polycan and calcium gluconate 2:98 mixtures.

BMD has been regarded as a valuable index to test the changes of bone quality in clinics especially to human, and they were generally and significantly decreased in periodontitis patient as progress of related alveolar bone losses[33)] and also in EPD[34),35)]. Once again, significant increases of maxillary BMD by treatment of Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula, in that order as compared with each Polycan and calcium gluconate single formula, but not by treatment of 1:1 and 9:1 mixtures, were considered as direct evidences that appropriated mixed formulation of Polycan and calcium gluconate showed synergic inhibitory effects against ligation-induced alveolar bone losses, and the fittest composition for further developing as effective agents to ameliorate periodontal diseases is Polycan and calcium gluconate 2:98 mixtures.

The importance of the acute inflammatory cell, mainly neutrophil infiltrations on gingival tissue in the evolution of periodontal disease has been demonstrated earlier[7),36)]. Although, inflammatory cells play a key roles for elimination of causes of inflammations[37)], activated neutrophils are also a potential source of oxygen metabolites[38)]. It is well established that oxygen metabolites play a role in the recruitment of neutrophils, preferentially neutrophils, into injured tissues[37)]. MPO is one of activating cytotoxic enzymes released from neutrophils[39)], and they were also markedly increases in periodontal diseases[26),40)]. The reduction of neutrophil influx into gingival tissue can be confirmed by MPO activity[26)]. In the present study, significant increases of MPO activities around ligation placed gingival tissues were also detected

in EPD control as compared with intact control, but significant decreases of gingival MPO activities were detected in all Polycan and calcium gluconate single or mixed formula treated rats as compared with EPD control, respectively. Especially, Polycan and calcium gluconate 1:9 mixed formula treated rats showed significant decreases of gingival MPO activities as compared with each Polycan and calcium gluconate single formula treated rats in first experiment, respectively. Moreover, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats also showed significant decreases of gingival MPO activities as compared with each Polycan and calcium gluconate single formula treated rats in second experiment, in that orders, suggesting appropriated mixed formulation of Polycan and calcium gluconate showed synergic anti-inflammatory effects against ligation-induced EPD, and the fittest composition for further developing as effective agents to ameliorate periodontal diseases is Polycan and calcium gluconate 2:98 mixtures.

The importance of pro-inflammatory cytokines, particularly TNF-α on periodontal disease has been documented[41),42)]. The cytokine TNF-α, produced by a variety of cell types, including splenocytes, was found to be associated with critical events leading to T-lineage commitment and differentiation[43)]. TNF-α may potentiate periodontitis by stimulating the release of eicosanoids and other cytokines, and activates neutrophils and macrophages, increasing the production and the release of reaction oxygen species and nitric oxide, which has been implicating in local tissue damage[44)]. Therefore, the inhibition of these cytokines could be contributing to the reduction of neutrophil infiltration, bone and cementum destruction[5),6)]. In this experiment, significant decreases of gingival TNF-α were detected in all test substance treated rats as compared with EPD control, respectively. Once again, as direct evidences that appropriated mixed formulation of Polycan and calcium gluconate showed synergic anti-inflammatory effects on the pro-inflammatory cytokine releases and the fittest composition for further developing as effective agents to ameliorate periodontal diseases is Polycan and calcium gluconate 2:98 mixtures, Polycan and calcium gluconate 2:98, 4:96, 8:92 and 10:90 mixed formula treated rats showed significant decreases of gingival TNF-α contents as compared with each Polycan and calcium gluconate single formula treated rats, in that orders, but not by treatment of 1:1 and 9:1 mixtures.

V. Conclusion

The results obtained in this study suggest that appropriated mixtures of Polycan and calcium gluconate showed synergic inhibitory effects against ligation-induced EPD and related alveolar bone losses in rats. Moreover, Polycan and calcium gluconate 2:98 showed the highest efficacies in this experiment, suggesting the fittest composition for further developing as effective agents to ameliorate periodontal diseases.

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References

1. Chambrone LA, Chambrone L. Tooth loss in well-maintained patients with chronic periodontitis during long-term supportive therapy in Brazil. Journal of clinical periodontology. 2006;33(10):759-764.

2. Sallay K, Sanavi F, Ring I, Pham P, Behling UH, Nowotny A. Alveolar bone destruction in the immunosuppressed rat. Journal of periodontal research. 1982;17(3):263-274.
3. Samejima Y, Ebisu S, Okada H. Effect of infection with *Eikenella corrodens* on the progression of ligature-induced periodontitis in rats. Journal of periodontal research. 1990;25(5):308-315.
4. Lohinai Z, Benedek P, Fehér E, Györfi A, Rosivall L, Fazekas A, Salzman AL, Szabó C. Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. British journal of pharmacology. 1998;123(3):353-360.
5. Ku SK, Cho HR, Sung YS, Kang SJ, Lee YJ. Effects of calcium gluconate on experimental periodontitis and alveolar bone loss in rats. Basic & clinical pharmacology & toxicology. 2011;108(4):241-250.
6. Kim YS, Kang SJ, Kim JW, Cho HR, Moon SB, Kim KY, Lee HS, Han CH, Ku SK, Lee YJ. Effects of Polycan, a β-glucan, on experimental periodontitis and alveolar bone loss in Sprague-Dawley rats. Journal of periodontal research. 2012;47(6):800-810.
7. Menezes AM, Rocha FA, Chaves HV, Carvalho CB, Ribeiro RA, Brito GA. Effect of sodium alendronate on alveolar bone resorption in experimental periodontitis in rats. Journal of periodontology. 2005;76(11):1901-1909.
8. Seto H, Ninomiya M, Nagata T. Alveolar bone resorption in animal models of periodontitis. Clinical calcium. 2006;16(2):248-254.
9. Schmeda-Hirschmann G, Yesilada E. Traditional medicine and gastroprotective crude drugs. Journal of ethnopharmacology. 2005;100(1-2):61-66.
10. Makkar A, Tewari S, Kishor K, Kataria S. An unusual clinical presentation of plasma cell gingivitis related to "Acacia" containing herbal toothpaste. Journal of Indian society of periodontology. 2013;17(4):527-530.
11. Seo HP, Kim JM, Shin HD, Kim TK, Chang HJ, Park BR, Lee JW. Production of -1,3/1,6-glucan by *Aureobasidium pullulans* SM-2001. Korean Journal of biotechnology and bioengineering. 2002;17 (4):376-380.
12. Song HB, Park DC, Do GM, Hwang SL, Lee WK, Kang HS, Park BR, Jang HJ, Son CW, Park EK, Kim SY, Huh TL. Effect of exopolymers of *Aureobasidium pullulans* on improving osteoporosis induced in ovariectomized mice. Journal of microbiology and biotechnology. 2006;16(1):37-45.
13. Shin HD, Yang KJ, Park BR, Son CW, Jang HJ, Ku SK. Antiosteoporotic effect of Polycan, beta-glucan from Aureobasidium, in ovariectomized osteoporotic mice. Nutrition. 2007;23(11-12):853-860.
14. Lee HS, Cho HR, Moon SB, Shin HD, Yang KJ, Park BR, Jang HJ, Kim LS, Ku SK. Effect of β-glucan from *Aureobsidium pullulans* on rat rib fracture healing. Laboratory animal research. 2008;24(1):39-44.
15. Kim HD, Cho HR, Moon SB, Shin HD, Yang KJ, Park BR, Jang HJ, Kim LS, Lee HS, Ku SK. Effects of β-glucan from *Aureobasidum pullulans* on acute inflammation in mice. Archives of pharmacal research. 2007;30(3):323-328
16. Kim HD, Cho HR, Moon SB, Shin HD, Yang KJ, Park BR, Jang HJ, Kim LS, Lee HS, Ku SK. Effect of Exopolymers from *Aureobasidum pullulans* on formalin-induced chronic paw inflammation in mice. Journal of microbiology and biotechnology. 2006;16(12):1954-1960.
17. Ku SK, Lee YJ, Lee SD, Cho HR, Moon SB, Kim KY, Kwon YS, Kim JW. Nephroprotective effect of Polycan on acute renal failure induced by cisplatin in rats. ISRN veterinary science. 2012;2012:862104.

18. Piller MB. Assessment of anti-inflammatory activity of calcium dobesilate. Effect on macrophages attaching to subcutaneously implanted cover slips in guinea pigs. Arzneimittelforchung. 1990;40(6):698-700.
19. Smith MM, Ghosh P, Numata Y, Bansal MK. The effects of orally administered calcium pentosan polysulfate on inflammation and cartilage degradation produced in rabbit joints by intraarticular injection of a hyaluronate-polylysine complex. Arthritis and rheumatism. 1994;37(1):125-136.
20. Bracken WM, Cuppage F, McLaury RL, Kirwin C, Klaassen CD. Comparative effectiveness of topical treatments for hydrofluoric acid burns. Journal of occupational medicine. 1985; 27(10):733-739.
21. Cavallini M, de Boccard F, Corsi MM, Fassati LR, Baruffaldi Preis FW. Serum pro-inflammatory cytokines and chemical acid burns in rats. Annals of burns and fire disasters. 2004; 17(2):84-87.
22. Karnad AS, Patil PA, Majagi SI. Calcium enhances anti-inflammatory activity of aspirin in albino rats. Indian journal of pharmacology. 2006;38(6):397-402.
23. Sohn KC, Kang SJ, Kim JW, Kim KY, Ku SK, Lee YJ. Effects of calcium gluconate, a water soluble calcium salt on the collagen-induced DBA/1J mice rheumatoid arthritis. Biomolecules & therapeutics. 2013;24(4):290-298.
24. Crawford JM, Taubman MA, Smith DJ. The natural history of periodontal bone loss in germfree and gnotobiotic rats infected with periodontopathic microorganisms. Journal of periodontal research. 1978;13(4):316-325.
25. Leitão RF, Ribeiro RA, Chaves HV, Rocha FA, Lima V, Brito GA. Nitric oxide synthase inhibition prevents alveolar bone resorption in experimental periodontitis in rats. Journal of periodontology. 2005;76(6):956-963.
26. Botelho MA, Rao VS, Carvalho CB, Bezerra-Filho JG, Fonseca SG, Vale ML, Montenegro D, Cunha F, Ribeiro RA, Brito GA. *Lippia sidoides* and *Myracrodruon urundeuva* gel prevents alveolar bone resorption in experimental periodontitis in rats. Journal of ethnopharmacology. 2007;113(3):471-478.
27. Bradley PP, Christensen RD, Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood. 1982;60(3):618-622.
28. Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ. Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. British journal of pharmacology. 1995;115(7):1265-1275.
29. Cunha FQ, Boukili MA, da Motta JI, Vargaftig BB, Ferreira SH. Blockade by fenspiride of endotoxin-induced neutrophil migration in the rat. European journal of pharmacology. 1993;238(1):47-52.
30. Levene A. Pathological factors influencing excision of tumours in the head and neck. Part I. Clinical otolaryngology and allied sciences. 1981;6(2):145-151.
31. Ludbrook J. Update: microcomputer statistics packages. A personal view. Clinical and experimental pharmacology & physiology. 1997;24 (3-4):294-296.
32. Tani-Ishii N, Minamida G, Saitoh D, Chieda K, Omuro H, Sugaya A, Hamada N, Takahashi Y, Kiyohara S, Kashima I, Teranaka T, Umemotot T. Inhibitory effects of incadronate on the progression of rat experimental periodontitis by porphyromonas gingivalis infection. Journal of periodontology. 2003;74(5):603-609.
33. Takaishi Y, Okamoto Y, Ikeo T, Morii H, Takeda M, Hide K, Arai T, Nonaka K. Correlations between periodontitis and loss of mandibular bone in relation to systemic bone

changes in postmenopausal Japanese women. Osteoporosis international. 2005;16(12):1875-1882.
34. Liu YF, Wu LA, Wang J, Wen LY, Wang XJ. Micro-computerized tomography analysis of alveolar bone loss in ligature- and nicotine-induced experimental periodontitis in rats. Journal of periodontal research. 2010;45(6):714-719.
35. Boas Nogueira AV, Chaves de Souza JA, Kim YJ, Damião de Sousa-Neto M, Chan Cirelli C, Cirelli JA. Orthodontic force increases interleukin-1β and tumor necrosis factor-α expression and alveolar bone loss in periodontitis. Journal of periodontology. 2013;84(9):1319-1326.
36. Liu H, Pope RM. Phagocytes: mechanisms of inflammation and tissue destruction. Rheumatic Diseases Clinics of North America. 2000;30(1):19-39.
37. Zimmerman BJ, Grisham MB, Granger DN. Role of oxidants in ischemia/reperfusion-induced granulocyte infiltration. The American journal of physiology. 1990;258(2 Pt 1):G185-190.
38. Sullivan GW, Sarembock IJ, Linden J. The role of inflammation in vascular diseases. Journal of leukocyte biology. 2000;67(5):591-602.
39. Işeri SO, Sener G, Yüksel M, Contuk G, Cetinel S, Gedik N, Yegen BC. Ghrelin against alendronate-induced gastric damage in rats. The Journal of endocrinology. 2005;187(3):399-406.
40. Holanda Pinto SA, Pinto LM, Cunha GM, Chaves MH, Santos FA, Rao VS. Anti-inflammatory effect of alpha, beta-Amyrin, a pentacyclic triterpene from *Protium heptaphyllum* in rat model of acute periodontitis. Inflammopharmacology. 2008;16(1):48-52.
41. Lima V, Bezerra MM, de Menezes Alencar VB, Vidal FD, da Rocha FA, de Castro Brito GA, de Albuquerque Ribeiro R. Effects of chlorpromazine on alveolar bone loss in experimental periodontal disease in rats. European journal of oral sciences. 2000;108(2):123-129.
42. Lima V, Vidal FD, Rocha FA, Brito GA, Ribeiro RA. Effects of tumor necrosis factor-alpha inhibitors pentoxifylline and thalidomide on alveolar bone loss in short-term experimental periodontal disease in rats. Journal of periodontology. 2004;75(1):162-168.
43. Samira S, Ferrand C, Peled A, Nagler A, Tovbin Y, Ben-Hur H, Taylor N, Globerson A, Lapidot T. Tumor necrosis factor promotes human T-cell development in nonobese diabetic/severe combined immunodeficient mice. Stem cells. 2004;22(6):1085-1100.
44. Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. Journal of immunology. 1998;160(1):403-409.