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Effects of Polycan, a β-glucan, on experimental periodontitis and alveolar bone loss in Sprague-Dawley rats

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Background and Objective: Polycan is a promising candidate for the treatment of periodontal disease. This study was undertaken to examine whether Polycan, a type of β-glucan, has a protective effect on ligature-induced experimental periodontitis and related alveolar bone loss in Sprague-Dawley rats.

Material and Methods: Polycan was orally administered, daily, for 10 d, at 21.25, 42.5 or 85 mg/kg, beginning 1 d after ligation. Changes in body weight and alveolar bone loss were monitored, and the anti-inflammatory effects of Polycan were determined by measuring the levels of myeloperoxidase (MPO), interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in gingival tissue. We also evaluated inducible nitric oxide synthase (iNOS) activity and malondialdehyde (MDA) concentrations as a measure of the antioxidant effect.

Results: Ligature placement led to a marked decrease in body weight, increased alveolar bone loss and increased concentrations of MPO, IL-1β, TNF-α and MDA, as well as increased iNOS activity and inflammatory cell infiltration and decreased collagen-fiber content. Histological examination revealed increases in the number and activity of osteoclast cells, decreases in alveolar bone volume and elevated percentages of osteclasts on the alveolar bone surface. Daily oral treatment with 42.5 or 85 mg/kg of Polycan for 10 d led to significant, dose-dependent inhibition of the effect of ligature placement.

Conclusion: Taken together, these results suggest that 10 d of oral treatment with Polycan effectively inhibits ligature placement-induced periodontitis and related alveolar bone loss via an antioxidant effect.

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Although inflammation is a pivotal response for protecting organisms against physical, chemical and infectious insults, an overactive inflammatory reaction can damage normal tissues and lead to symptoms such as fever and pain (1). Given that chronic inflammation is a long-lasting inflammatory response, in periodontal disease chronic inflammation is seen either as gingivitis (in its mild form) or as periodontitis (in its advanced form). Periodontitis is characterized by periodontal pocket formation, connective tissue detachment and alveolar bone

resorption, ultimately resulting in tooth loss (2,3).

Recent studies have shown that bacteria, toxins, enzymes and metabolites in dental plaque are primary factors in the development of periodontitis and play an important role in the initiation of the inflammatory process (4). Destructive processes concomitant with the inflammatory response are mainly responsible for the tissue damage. Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1 are vital for the initiation of an inflammatory response and consequently tissue destruction (5,6). TNF-α may exacerbate periodontitis by stimulating the release of eicosanoids and other cytokines, such as IL-1 (7). IL-1 activates neutrophils and macrophages, increasing the production and release of reactive oxygen species (ROS) and nitric oxide (NO), which have been implicated in local tissue damage (8).

It is well documented that oxidative stress caused by the production of excessive amounts of ROS is involved in the pathogenesis of periodontitis (9). In addition, NO and inducible nitric oxide synthase (iNOS) damage alveolar bones and surrounding tissue in periodontal disease (10,11). Oxygen metabolites contribute to the recruitment of neutrophils, particularly polymorphonuclear cells (PMNs), into injured tissues (12). Activated PMNs, a source of oxygen metabolites (13), result in the release of myeloperoxidase (MPO), which is a cytotoxic enzyme (14) and is markedly increased in periodontal disease (15–17). ROS can also stimulate lipid peroxidation to form malondialdehyde (MDA), which is elevated in periodontal disease (11,18).

β-glucans are fiber-type complex sugars (polysaccharide) derived from the cell walls of baker's yeast, oat and barley fiber, and many medicinal mushrooms (19). β-glucan is an effective immunomodulator with anti-inflammatory properties, and regulates the expression of IL-1, IL-10 and interferon-gamma in the peritonitis model (20). The active component of β-glucan from *Ganoderma lucidum* induces the production of TNF-α and (with lipopolysaccharide) induces TNF-α production, suggestive of immunostimulatory activity (21). Şener *et al*. (22) reported that β-glucan ameliorated methotrexate-induced oxidative damage via its antioxidant effects (22). Polycan, a purified β-glucan from *Aureobasidium pullulans* SM-2001, is comprised mostly of β-1,3/1,6-glucan and other organic materials, such as amino acids, monounsaturated or diunsaturated fatty acids (linoleic and linolenic acids) and fibrous polysaccharides (23). Polycan has anti-osteoporotic effects (23,24), inhibiting bone loss and accelerating bone formation (25) through anti-inflammatory activities (26,27).

Accordingly, Polycan is a promising candidate for the treatment of periodontal disease. However, there are no studies examining the effects of Polycan on experimental periodontitis or related alveolar bone loss. Thus, we performed a comparative study of Polycan and indomethacin on ligature-induced experimental periodontitis and alveolar bone loss. This animal model is most frequently used for the induction of periodontal disease by ligature placement. This experimental periodontitis model has the advantage of inducing periodontitis and alveolar bone loss similarly to naturally acquired human periodontal disease (3,28). Indomethacin was selected as a reference drug because it is a well-studied, nonselective cyclooxygenase inhibitor, nonsteroidal anti-inflammatory drug (29,30) that can slow the progression of experimental periodontitis in rats (31). To determine the effects of Polycan on periodontitis, we used histopathological analyses and measured the levels of IL-1β and TNF-α. In addition, iNOS activity, lipid peroxidation and MPO levels were determined.

Material and methods

Animals and husbandry

Forty-two male Sprague-Dawley rats (160–180 g; 6 wk of age upon receipt; Japan SLC Inc., Hamamatsu, Shizuoka, Japan) were used in this study after 9 d of acclimatization. The rats were housed, three or four per polycarbonate cage, in a temperature (20–25°C) and humidity (40–45%) controlled room. The light : dark cycle was 12 h : 12 h, and a normal rodent pellet diet and water were supplied freely throughout all experimental periods, including acclimatization. All animals were fasted overnight (about 18 h with *ad libitum* access to water) before they were killed and were treated according to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Science, 1996 National Research Council, Washington D.C. Experimental periodontal disease was induced in 35 rats by ligature placement, and the remaining seven rats were used as intact controls. The experimental groups (six groups, seven rats per group) were as follows: intact control (distilled water, orally administered vehicle control); experimental periodontitis control (experimental periodontal disease-induced and vehicle-treated control); indomethacin (experimental periodontitis-induced reference drug group treated with 5 mg/kg of indomethacin); Poly 21.25 (experimental periodontitis-induced experimental group treated with 21.25 mg/kg of Polycan); Poly 42.5 (experimental periodontitis-induced experimental group treated with 42.5 mg/kg of Polycan); and Poly 85 (experimental periodontitis-induced experimental group treated with 85 mg/kg of Polycan).

Preparation and administration of test materials

Polycan was supplied by Glucan Corp. (Pusan, Korea) as a 1.7% (Brix) stock solution. Indomethacin was purchased from Fluka (St. Gallen, Switzerland). All test materials were stored in a refrigerator (4°C) for protection from light and moisture. Three different concentrations of Polycan were prepared by dilution of a 1.7% stock solution (2.125, 4.25 and 8.5 mg/5 mL) using distilled water as a vehicle, and 5 mg of indomethacin were dissolved in 5 mL of distilled water. Test materials were administered orally in a volume of 5 mL/kg, once a day for 10 d, from 24 h after ligature placement.

Induction of experimental periodontitis

A sterilized nylon (3–0) thread ligature was placed around the cervix of the second left-upper molar of rats anesthetized with an intraperitoneal injection of a 25 mg/kg mixture of Zoletile (Zoletile 50; Virbac Laboratories, Carros cedex, France), as described previously (3). The ligature was knotted on the buccal side of the tooth, resulting in a palatial subgingival position and a buccal supragingival position.

Changes in body weight

Body weight was measured using an automatic electronic balance (Precisa Instrument, Dietikon, Switzerland), daily, for the duration of the experiment, beginning 1 d before ligature placement. To control for individual differences, body-weight gain after 10 d of drug administration was also calculated.

Measurements of alveolar bone loss

The rats were killed after 10 d of drug administration (i.e. at the end of the study period; 11 d after ligature placement), and the maxillary bone containing the ligature placement site (second molar) was excised, fixed in 10% neutral formalin and stained with a 1% aqueous methylene blue (Sigma, St Louis, MO, USA) solution prepared with tap water, to differentiate bone from teeth. Horizontal alveolar bone loss and the distance between the cusp tip and the alveolar bone were measured using a modification of the method of Crawford *et al.* (32), as described by Samejima *et al.* (2). Measurements were made along the axis of each root of the first (three roots), second (two roots) and third (two roots) molars. Total alveolar bone loss was obtained by summing the recordings from buccal tooth surfaces as mm/rats (16,17).

Detection of IL-1β and TNF-α in rat maxillary gingival tissue

The buccal gingival tissue from the area surrounding the ligature placement was collected 11 d after induction of experimental periodontitis. The tissues collected were homogenized and processed as described by Safieh-Garabedian *et al.* (33) and modified as described by Botelho *et al.* (16). The TNF-α and IL-1β concentrations were determined by ELISA, as described by Cunha *et al.* (34). Microtiter plates were coated overnight at 4°C with antibody against rat TNF-α or rat IL-1β (10 μg/mL). After blocking the plates, the samples and standards were added at various dilutions, in duplicate, and were incubated at 4°C for 24 h. The plates were washed three times with buffer. After washing the plates, 100 mL of biotinylated sheep polyclonal anti-rat TNF-α or anti-rat IL-1β (diluted 1 : 1000 with assay buffer containing 1% bovine serum albumin; Abcam, Cambridge, UK) was added to the wells. After further incubation at room temperature for 1 h, the plates were washed, and 100 μL of a 1 : 5000 dilution of avidin-horseradish peroxidase (Abcam, UK) was added. The color reagent *o*-phenylenediamine (100 μL; Sigma) was added after 15 min, and the plates were incubated in the dark at 37°C for 20 min. The enzyme reaction was stopped with H_2SO_4, and absorbance was measured at 490 nm.

Measurement of MPO activity

Gingival tissues around ligature placement were collected 11 d after induction of experimental periodontitis to measure neutrophil accumulation. A spectrophotometric assay described previously (35) was utilized to measure MPO activity. The buccal gingivae surrounding the upper left molars were removed and stored at −70°C. The material was suspended in 0.5% hexadecyltrimethylammonium bromide (Gibco, Grand Island, NY, USA) in 50 mM potassium phosphate buffer, pH 6.0, to solubilize the MPO. After homogenization (for 15 s) in an ice bath, the samples were subjected to two freeze–thaw cycles. Additional buffer was added to the test tube to give a final volume of 400 μL of buffer per 15 mg of tissue, and the tube was incubated for 12 min. After centrifugation at 1000 *g* for 12 min, 0.1 mL of the supernatant was added to 2 mL of phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/mL of *o*-dianisidine dihydrochloride (Sigma), distilled water and 0.0005% hydrogen peroxide to give a final volume of 2.1 mL per tube. The absorbance was measured spectrophotometrically (at 460 nm). One unit of activity was defined as that degrading 1 μmol of peroxide/min at 25°C. The results are expressed as MPO units/mL.

MDA measurement

The MDA level, as an indicator of lipid peroxidation (36), in the buccal gingival tissue was determined in the area surrounding the ligature, as previously described (37). Briefly, tissues were placed in a homogenization buffer composed of 50 mM Tris–HCl, 0.1 mM ethylene glycol tetraacetic acid (EGTA) and 1 mM phenylmethanesulfonyl fluoride (pH 7.4) and were then homogenized. Aliquots (100 μL) of the buccal gingival tissue homogenate were added to a reaction mixture containing 200 μL of 8.1% (w/v) sodium dodecyl sulfate (Sigma), 1500 μL of 20% (v/v) acetic acid (pH 3.5), 1500 μL of 0.8% (w/v) thiobarbituric acid (Sigma) and 700 μL of distilled water. The samples were then heated for 1 h at 95°C and centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 650 nm.

iNOS activity measurement

Gingival tissue iNOS activity was assessed as previously described (18). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates. Briefly, homogenates (30 mL) were incubated in the presence of [^{3}H]L-arginine (10 mM, 5 kBq per tube), nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 mM) and calcium (2 mM) for 30 min at 22°C. The reactions were stopped by dilution with 0.5 mL of ice-cold Hepes (pH 5.5) containing EGTA (2 mM) and ethylenediaminetetraacetic (EDTA) (2 mM).

Experiments performed in the absence of NADPH were conducted to determine the extent of [^{3}H]L-citrulline formation, independent of specific NOS activity. Experiments in the presence of nicotinamide adenine dinucleotide (NADH) without calcium and in the presence of EGTA (5 mM) determined the calcium-independent NOS activity. Reaction mixtures were applied to Dowex 50W (Na/form) columns, and the eluted [^{3}H]L-citrulline activity was measured in a liquid scintillation counter (Wallac, Gaithersburg, MD, USA).

Histopathology

The maxilla area surrounding the ligature placement was sampled 11 d after induction of experimental periodontitis and samples were fixed in 10% neutral-buffered formalin. After fixation, the maxilla was decalcified for 5 d using a decalcifying solution (24.4% formic acid and 0.5 M sodium hydroxide) (mixed decalcifying solution was exchanged once per day for 5 d). The maxilla was longitudinally trimmed (including first and second molars), embedded in paraffin, sectioned (3–4 μm) and stained with hematoxylin & eosin and Masson's trichrome stain (for collagen-fiber observations). The histological profiles of the gingival tissues and alveolar bones were observed and compared with those of the intact control group.

Histomorphometry

The numbers of infiltrated inflammatory cells (numbers/mm^2 of gingival tissues) and collagen-occupied regions (%/mm^2 of gingival tissues) on the gingival areas between the first and the second molars were measured using histomorphometrical analyses of prepared longitudinally trimmed samples using a digital image analyzer (DMI-300; DMI, Deagu, Korea). In addition, alveolar bone volumes (%/mm^2 of alveolar bone areas), osteoclast cell numbers (numbers/mm^2 of alveolar bone surface) and their percentage coverage (%/mm^2 of alveolar bone surface) were measured on the alveolar bone regions between the first and the second molars (roots of teeth were excluded) on prepared, longitudinally trimmed samples using a digital image analyzer. Interexaminer calibration was performed under blind conditions, whereby two histopathologists examined the same slide.

Statistical analyses

Multiple comparison tests for different dose groups were conducted. Homogeneity of variance was examined using Levene's test. If Levene's test indicated no significant deviations from homogeneity of variance, the data obtained were analyzed by one-way analysis of variance followed by a least-significant-differences multicomparison test to determine which pairs were significantly different. In cases in which significant deviations from homogeneity of variance were observed by Levene's test, the nonparametric Kruskal–Wallis *H* comparison test was conducted. When a significant difference was observed in the Kruskal–Wallis *H*-test, the Mann–Whitney *U*-Wilcoxon Rank Sum *W*-test was conducted to determine the specific pairs that were significantly different. Statistical analyses were conducted using SPSS for Windows (Release 12.0K; SPSS Inc., Armonk, NY, USA).

Results

Polycan affected experimental periodontitis-related changes in body weight

Compared with the intact control group, body weights were significantly decreased in the experimental periodontitis control group from 2 d after ligature placement (from 1 d after initiation of administration of test materials); the gains in body weight were significantly decreased during the 10-d period when test materials were administered. In contrast, the body weights of the rats in the Poly 42.5 and Poly 85 groups were significantly increased compared with the body weights of experimental periodontitis control group (from 7 and 8 d, respectively, after the start of administration of test materials). The gains in body weight were also remarkably increased in these groups compared with the experimental periodontitis control group during the 10-d period when test materials were administered.

A significant decrease in body weight was observed in the indomethacin-treated group from 5 d after initiation of administration of the drug. In addition, during the 10-d drug-administration period, body weight gains were decreased in the indomethacin-treated group compared with the experimental periodontitis control group (Table 1). Compared with the intact control group, body-weight gains in the experimental periodontitis control group were decreased by 20.38% during the 10-d drug-administration period. They were changed by −27.47%, 12.27%, 32.00% and 35.20% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group.

Polycan dramatically ameliorates alveolar bone loss in experimental periodontitis-induced rats

We observed increases of alveolar bone loss in the experimental periodontitis control group compared with the intact control group. Conversely, significant and dose-dependent decreases in alveolar bone loss were detected in two of the three Polycan dosage groups (Poly 42.5 and Poly 85) compared with the experimental periodontitis control group (Fig. 1). The distance from the cusp tip to the alveolar bone decreased by 43.26%, 7.45%, 27.44% and 61.25% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group.

Polycan significantly decreases TNF-α and IL-1β levels in gingival tissues

Gingival TNF-α and IL-1β levels were significantly increased in the experimental periodontitis control group compared with the intact control group. Figure 2A shows that the gingival TNF-α level was significantly decreased, in a dose-dependent manner in all three different Polycan dosage

Table 1. Body-weight gains during 10 d of continuous oral treatment with Polycan in experimental periodontitis-induced rats

Group	Body weight (g): Before	At ligation	At initiation of test article treatment (A)	At final administration of test article (B)	Body weight gain (B–A)
Controls					
Intact	238.71 ± 8.14	248.57 ± 5.39	257.14 ± 10.67	324.43 ± 17.49	67.29 ± 9.52
EPD	239.43 ± 6.92	251.86 ± 7.43	248.29 ± 11.74	301.86 ± 9.92[b]	53.57 ± 9.95[a]
Reference					
Indomethacin (5 mg/kg)	237.29 ± 8.14	248.57 ± 10.34	248.43 ± 12.63	287.29 ± 5.88[b,A]	38.86 ± 11.07[b,A]
Polycan-treated					
21.25 mg/kg	238.57 ± 8.54	250.00 ± 7.12	250.14 ± 7.36	310.29 ± 7.32[a]	60.14 ± 11.47
42.50 mg/kg	239.14 ± 6.54	251.71 ± 6.37	250.14 ± 7.24	320.86 ± 9.17[B]	70.71 ± 2.43[B]
85.00 mg/kg	237.71 ± 6.07	250.57 ± 6.40	246.29 ± 7.95	318.71 ± 11.37[A]	72.43 ± 11.27[B]

Values are expressed as mean ± standard deviation from seven rats per group.
Experimental periodontal disease (EPD) was induced by ligature placement on the cervix of the upper-left second molar.
[a]$p < 0.05$.
[b]$p < 0.01$ compared with the intact control group.
[A]$p < 0.05$.
[B]$p < 0.01$ compared with the EPD control gtoup.

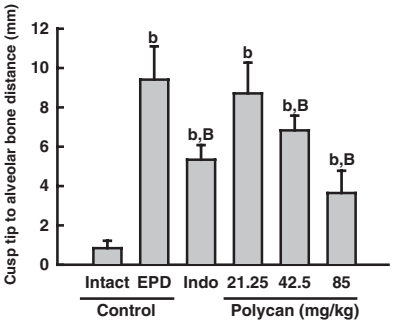

Fig. 1. Polycan ameliorated alveolar bone loss in experimental periodontitis-induced rats. Animals were killed after 10 d of drug administration, and the maxillary bone containing the ligature placement site (second molar) was used to determine the difference between bone and teeth. Values are expressed as mean ± standard deviation of seven rats. EPD, experimental periodontal disease induced by ligature placement on the cervix of the upper-left second molar; Indo, group treated with 5 mg/kg of indomethacin. [b]$p < 0.01$ compared with the intact control group; [B]$p < 0.01$ compared with the experimental periodontitis control group.

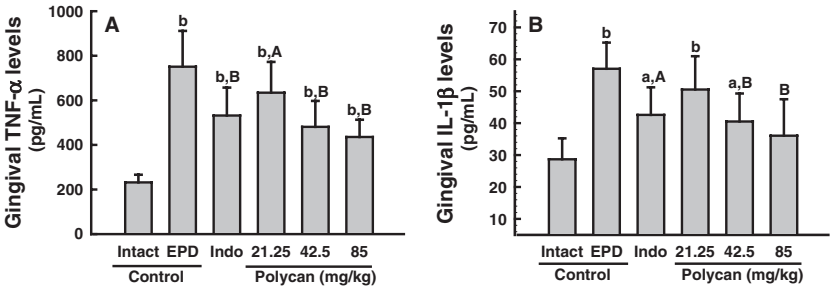

Fig. 2. Polycan exerts a protective effect on experimental periodontitis-induced rats by inhibiting an increase in tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) in gingival tissues. (A, B) Gingival tissue from the area surrounding the ligature placement was collected 11 d after experimental periodontitis induction. The amounts of TNF-α and IL-1β were measured using ELISAs. Values are expressed as mean ± standard deviation of seven rats. EPD, experimental periodontal disease induced by ligature placement on the cervix of the upper-left second molar; Indo, group treated with 5 mg/kg of indomethacin. [a]$p < 0.05$ and [b]$p < 0.01$ compared with the intact control group; [A]$p < 0.05$ and [B]$p < 0.01$ compared with the experimental periodontitis control group.

groups compared with the experimental periodontitis control group. The gingival TNF-α level of the experimental periodontitis control group increased by 223.91% compared with that of the intact control group. The gingival TNF-α level was also decreased by 29.18%, 15.52%, 35.93% and 41.98% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group (Fig. 2A). Figure 2B indicates that significant and dose-dependent decreases in the gingival IL-1β level occurred in the groups receiving higher dosages of Polycan (42.5 and 85 mg/kg) compared with the experimental periodontitis control group. The gingival IL-1β level in the experimental periodontitis control group was increased by 98.87% compared with the intact control group and was decreased by 25.33%, 11.39%, 28.99% and 36.77% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group (Fig. 2B).

Polycan diminished MPO activity in gingival tissues

Significant increases in the gingival MPO level were detected in the experimental periodontitis control group compared with the intact control group. However, significant and dose-dependent decreases in the gingival MPO level were detected in two of the groups treated with Polycan (Poly 42.5

and Poly 85) compared with the experimental periodontitis control group. The gingival MPO level in the experimental periodontitis control group was increased by 320.42% compared with the intact control group and was decreased by 48.20%, 13.33%, 34.67% and 51.81% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group (Fig. 3A).

Polycan reduced iNOS activity in gingival tissues

Gingival iNOS activity was significantly increased in the experimental periodontitis control group compared with the intact control group. However, significant and dose-dependent decreases in gingival iNOS activity were detected in the two groups treated with higher dosages of Polycan (42.5 and 85 mg/kg) compared with the experimental periodontitis control group (Fig. 3B). Gingival iNOS activity in the experimental periodontitis control was increased by 493.10% compared with the intact control group and was decreased by 23.09%, 13.00%, 28.18% and 59.36% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group.

Polycan decreases iNOS activity in gingival tissues

A significant ($p < 0.01$) increase in the gingival MDA level was observed in the experimental periodontitis control group compared with the intact control group. However, significant ($p < 0.01$) and dose-dependent decreases in the gingival MDA level were detected in the two groups treated with higher dosages of Polycan (42.5 and 85 mg/kg) compared with the experimental periodontitis control group (Fig. 3C). The gingival MDA level in the experimental periodontitis control group increased by 353.23% compared with the intact control group and decreased by 24.30%, 11.63%, 28.91% and 40.20% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group.

Polycan affects experimental periodontitis-induced histopathological changes

The number of inflammatory cells (mainly PMNs) infiltrating the gingival tissues in the experimental periodontitis control group increased by 10528.95% compared with the intact control group. Conversely, it decreased by 81.07%, 22.25%, 59.11% and 89.29% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group. The collagen fiber-occupied region in the gingival tissues of the experimental periodontitis control decreased by 55.97% compared with the intact control group. In contrast, the collagen fiber-occupied region increased by 92.29%, 14.63%, 71.67%, and 104.64% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group. The alveolar bone volume decreased by 49.72% in the experimental periodontitis control group compared with the intact control group. However, it increased by 31.45%, 12.05%, 48.15% and 76.38% in the indomethacin, Poly 21.25, Poly 42.5, and Poly 85 groups, respectively, compared with the experimental periodontitis control group. Compared with the intact control group, the number of osteoclast cells on the alveolar bone surface showed an increase of 600.00% in the experimental periodontitis control group. However, it decreased by 16.27%, 14.29%, 33.33% and 48.02% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group. The percentage of osteoclast cell-occupied regions on the alveolar bone surface in the experimental periodontitis control group increased by 738.97% compared with the intact control group. The percentage of osteoclast

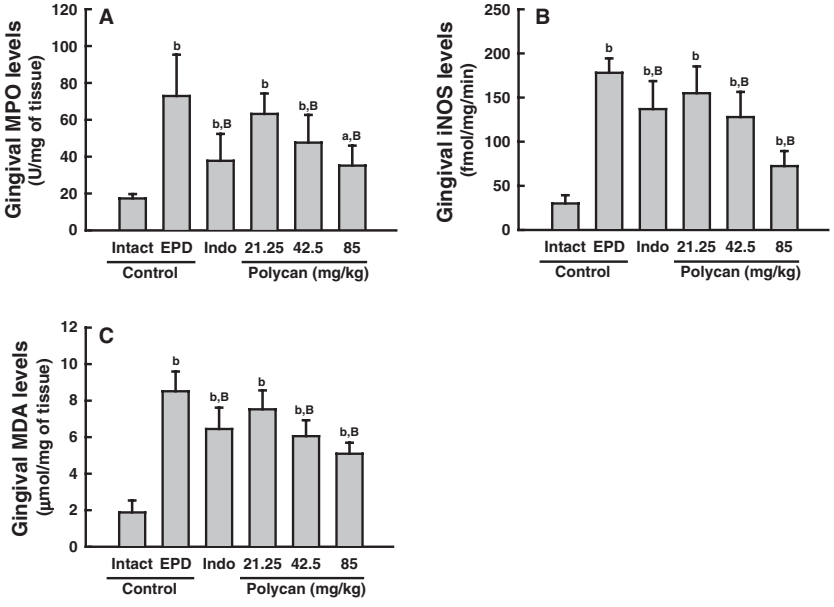

Fig. 3. Polycan decreased the levels of myeloperoxidase (MPO), malondialdehyde (MDA) and inducible nitric oxide synthase (iNOS) activity in gingival tissues. (A–C) Gingival tissues from the area surrounding the ligature placement were collected 11 d after experimental periodontitis induction and were used to measure the levels of MPO, MDA and iNOS. Values are expressed as mean ± standard deviation of seven rats. EPD, experimental periodontal disease induced by ligature placement on the cervix of the upper-left second molar; Indo, group treated with 5 mg/kg of indomethacin. $^{a}p < 0.05$ and $^{b}p < 0.01$ compared with the intact control group; $^{B}p < 0.01$ compared with the experimental periodontitis control group.

Table 2. Histomorphometric analysis of maxillary regions (gingival and alveolar bone areas) after 10 d of continuous oral treatment with Polycan in experimental periodontitis-induced rats

Group	Gingival areas between ligatured first and second molars		Alveolar bone areas around the roots of ligatured first and second molars		
	Inflammatory cell infiltration (n/mm^2)	Collagen fiber-occupied (%/mm^2)	Alveolar bone volume (%)	Osteoclast cell number (n/mm of alveolar bone surface)	Osteoclast cell percentage (%/mm of alveolar bone surface)
Controls					
Intact	10.86 ± 3.44	81.90 ± 8.61	75.33 ± 6.05	5.14 ± 2.54	3.85 ± 2.41
EPD	1154.00 ± 415.70^{b}	36.06 ± 8.82^{b}	37.87 ± 6.46^{b}	36.00 ± 8.41^{b}	32.32 ± 8.92^{b}
Reference					
Indomethacin (5 mg/kg)	218.43 ± 169.26^{b,B}	69.34 ± 6.91^{b,B}	49.79 ± 9.15^{b,B}	30.14 ± 12.24^{b}	27.44 ± 8.99^{b}
Polycan-treated					
21.25 mg/kg	897.29 ± 529.65^{b}	41.34 ± 9.48^{b}	42.44 ± 3.75^{b}	30.86 ± 9.32^{b}	27.80 ± 6.14^{b}
42.50 mg/kg	471.86 ± 237.92^{b,B}	61.90 ± 10.64^{b,B}	56.11 ± 11.75^{b,B}	24.00 ± 6.68^{b,A}	12.98 ± 6.78^{a,B}
85.00 mg/kg	123.57 ± 58.07^{b,B}	73.79 ± 8.11^{B}	66.80 ± 6.68^{b,B}	18.71 ± 1.80^{b,B}	9.70 ± 2.73^{b,B}

Values are expressed as mean ± standard deviation from seven rats in each group. Experimental periodontal disease (EPD) was induced by ligature placement on the cervix of the upper-left second molar.
$^{a}p < 0.05$.
$^{b}p < 0.01$ compared with the intact control group.
$^{A}p < 0.05$.
$^{B}p < 0.01$ compared with the EPD control group.

cell-occupied regions changed by −15.10%, −14.00%, −59.86% and −69.99% in the indomethacin, Poly 21.25, Poly 42.5 and Poly 85 groups, respectively, compared with the experimental periodontitis control group (Table 2, Figs 4 and 5).

Discussion

This is the first study to demonstrate a protective effect of Polycan on ligature-induced experimental periodontitis and related alveolar bone loss. Our results showed that Polycan treatment dramatically increased body weight and weight gain compared with the experimental periodontitis control group. In addition, alveolar bone loss was significantly decreased in a dose-dependent manner in Polycan-treated Sprague-Dawley rats. Because periodontitis and related alveolar bone loss directly induced mal-mastication, resulting in body-weight loss in experimental periodontitis, it is possible that the inhibition of experimental periodontitis-related decreases in body weight is associated with the alleviation of periodontitis and related alveolar bone loss.

In an attempt to demonstrate the effect of Polycan on periodontitis in further detail, IL-1β and TNF levels were monitored because IL-1β and TNF are considered as risk factors for periodontitis. We showed that Polycan exerts its inhibitory effect on TNF-α and IL-1β production in gingival tissues. In other words, the progression of periodontal disease can be delayed by antagonists to specific host mediators such as TNF and IL-1. TNF and IL-1 induce the expression of other mediators that amplify the inflammatory response, enhance the production of lytic enzymes and stimulate the production of chemokines, ultimately resulting in tissue destruction (5,6). These proinflammatory mediators also activate pathways that consequently lead to the destruction of gingival tissue through the elaboration of non-specific proteases and matrix metalloproteinases. Furthermore, TNF and IL-1 act synergistically to stimulate bone resorption (6). Therefore, inhibition of these cytokines could contribute to the reduction of neutrophil infiltration, leading to the destruction of bone and cementum (16). Growing evidence suggests that the production of elevated levels of TNF/IL-1 may be a pivotal factor in periodontal disease development based upon their increased expression in inflamed gingivae and relatively high levels in the gingival cervicular fluid of periodontitis patients (6,38–40). Both *in vivo* and epidemiology studies have reported that antagonists to TNF can delay disease progression (41,42).

We observed increases in iNOS activity and MDA levels around the ligature placed in gingival tissues. However, Polycan treatment inhibited the increase in iNOS activity and MDA levels in a dose-dependent manner. Thus, it is possible that Polycan ameliorates the damaged tissues in experimental periodontitis through inhibition of iNOS activity. It is likely that increased levels of ROS are involved in the pathogenesis of periodontitis (10) and are common to both bacterial and host-mediated tissue-damage pathways. A number of studies have proposed that the effect is caused by an imbalance between oxidant and antioxidant activity observed in saliva and gingival crevicular fluid (9,43). NO acts as an important mediator in a variety of physiological and pathophysiological processes (37,44,45). iNOS, a distinct isoform of NOS, can be induced by proinflammatory agents such as endotoxins, IL-1β, TNF-α and interferon-gamma in a variety of cells. Enhanced production of NO following

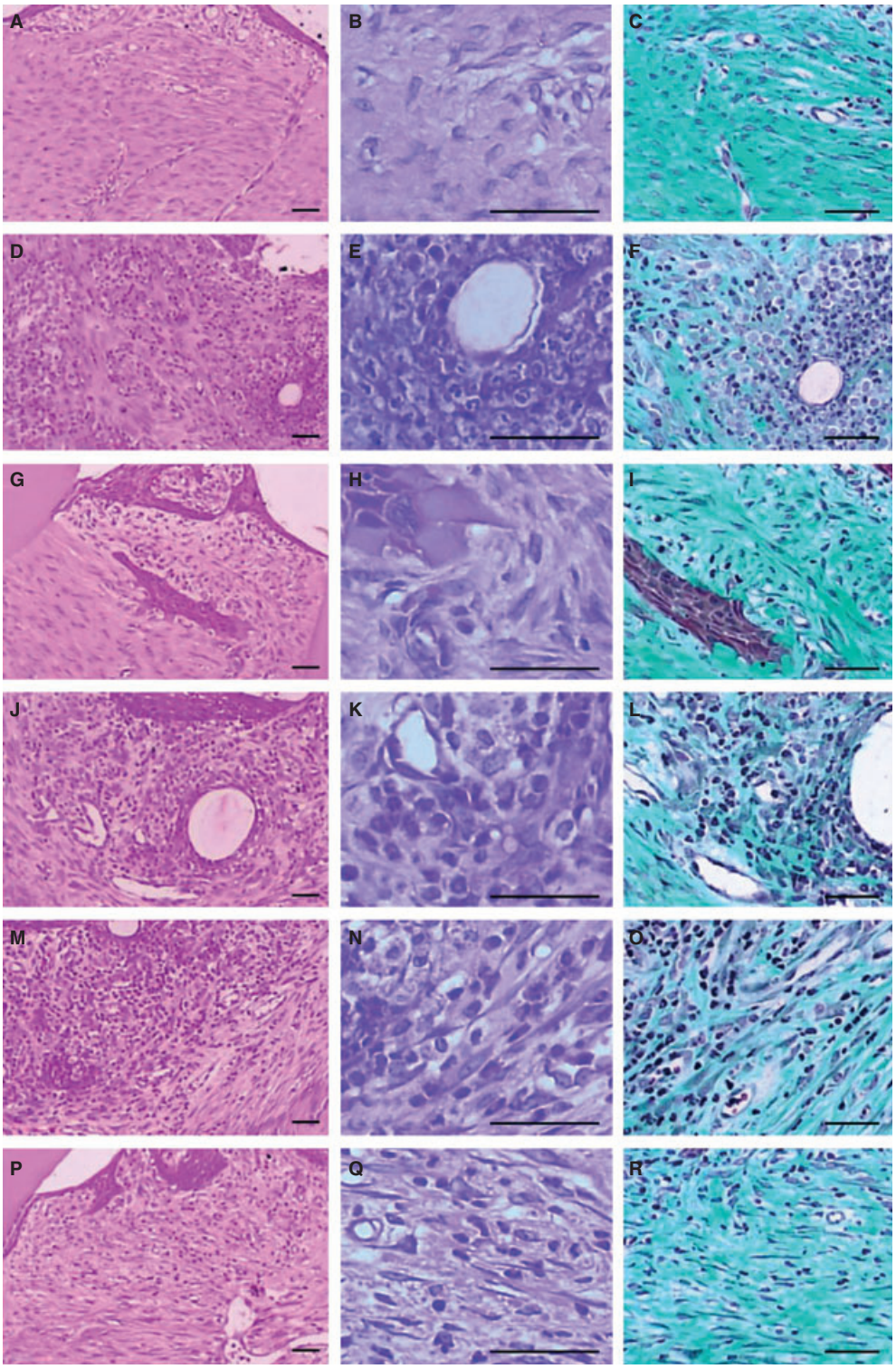

Fig. 4. Polycan induced histopathological changes in gingival tissues between the first and second molars in the intact vehicle control group (A–C), in the experimental periodontitis control group (D–F), in the Ind group (G–I) and in the Poly 21.25 (J–L), Poly 42.5 (M–O) and Poly 85 (P–R) groups. EPD, experimental periodontal disease induced by ligature placement on the cervix of the upper-left second molar; Ind group, group treated with 5 mg/kg of indomethacin; Poly 21.25 group, group treated with 21.25 mg/kg of Polycan; Poly 42.5 group, group treated with 42.5 mg/kg of Polycan; Poly 85 group, group treated with 85 mg/kg of Polycan. A, B, D, E, G, H, J, K, M, N, P, Q: hematoxylin and eosin stain; C, F, I, L, O, R: Masson's trichrome stain. Scale bars = 80 μm.

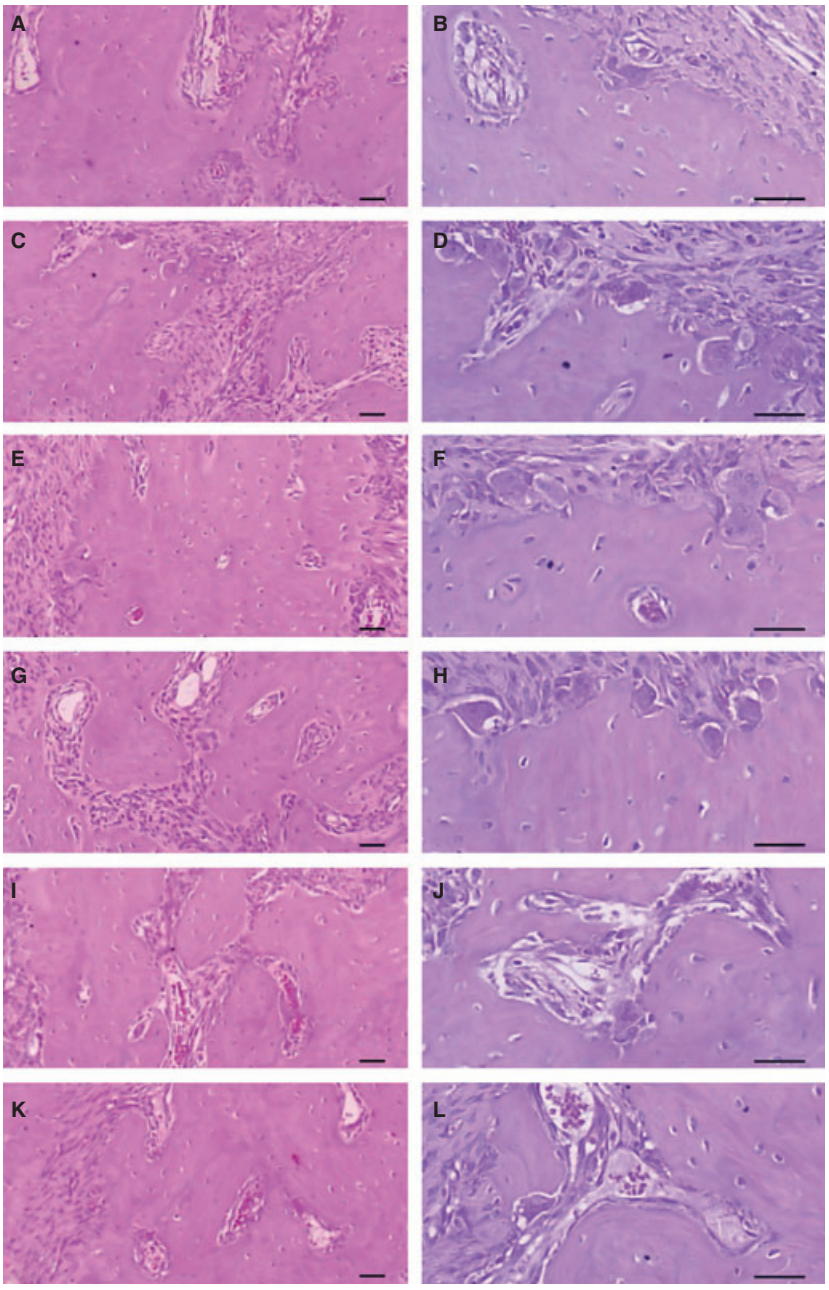

Fig. 5. Polycan induced histopathological changes in alveolar bone areas between the first and the second molars in the intact vehicle control group (A, B), experimental periodontitis control group (C, D), Ind group (E, F), and Poly 21.25 (G, H), Poly 42.5 (I, J) and Poly 85 (K, L) groups. EPD, experimental periodontal disease induced by ligature placement on the cervix of the upper-left second molar; Ind group, group treated with 5 mg/kg of indomethacin; Poly 21.25 group, group treated with 21.25 mg/kg of Polycan; Poly 42.5 group, group treated with 42.5 mg/kg of Polycan; Poly 85 group, group treated with 85 mg/kg of Polycan. All sections were stained with hematoxylin and eosin. Scale bars = 80 μm.

the induction of iNOS has been implicated in inflammation (46–48). In periodontal disease, activation of iNOS and related molecules increases NO production, damaging the surrounding tissues (especially in alveolar bone) (49,50). Experimental periodontitis-induced oxidative stress was determined indirectly by quantifying MDA accumulation in tissue. Many antioxidants are known to have partially or fully favorable effects on periodontitis and related alveolar bone loss (18,50–52).

Gingival MPO levels were significantly increased in the experimental periodontitis control group, whereas Polycan inhibited these increases in a dose-dependent manner, suggesting that Polycan inhibits the cytotoxic effects of PMNs. It is well known that oxygen metabolites play a role in the recruitment of neutrophils, preferentially PMNs, to injured tissues (12). Infiltration of acute inflammatory cells (primarily neutrophils) into gingival tissue plays an important role in the development of periodontal disease (3). The reduction of neutrophil influx into gingival tissue can be confirmed by reduced MPO activity (16).

In this study, we used the ligature model to induce experimental periodontitis in rats. There are many methods to induce experimental periodontitis, such as dietary manipulation, introduction of pathogenic microorganisms and placement of a ligature (53–56). Of these methods, inducing experimental periodontitis in a rat by placing a ligature around the cervix of the mandibular first molar tooth is an approved and highly predictable technique for evaluating drugs against periodontal diseases. However, because of the limitation that ligature-induced periodontitis does not mimic natural disease, further studies needs to be performed to clarify the effect of Polycan against periodontal disease induced by microorganisms-.

Together with previous experimental periodontitis studies (15,30), this study confirms that periodontitis results in marked inflammatory cell infiltration and edematous changes in the gingival tissues between the first and second molars where the ligature is placed. We can also identify alveolar bone absorption as a result of the activation of osteoclast cells. In addition, increases in the infiltration of inflammatory cells, including neutrophils, were detected. Based on inflammatory cell infiltration and alveolar bone damage (16,31,32), the histological changes were exacerbated, concomitant with decreases in collagen-occupied regions

related to edematous changes, decreases in bone volume and increases in osteoclast cell number and their percentages on alveolar bone surfaces. These histopathological changes related to periodontitis and alveolar bone loss were inhibited, in a Polycan dose-dependent manner, in Polycan-treated rats. Therefore, Polycan may have a protective effect on ligature-induced periodontitis and play a role in alveolar bone loss.

Taken together, these results suggest that oral administration of Polycan ameliorates ligature placement-induced periodontitis by reducing the amounts of proinflammatory cytokines and inhibiting the production of oxidative stress, which recruits inflammatory cells into the tissue and decreases alveolar bone loss. Thus, Polycan is a promising drug for the treatment of periodontitis.

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