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Effects of topical application of inorganic polyphosphate on tissue remodeling in rat inflamed gingiva

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Background and Objective: Inorganic polyphosphate [poly(P)] is a biopolymer found in almost all cells and tissues, and which promotes tissue remodeling. However, there is limited information on how poly(P) affects the connective tissue in inflamed gingiva. This study examined the effects of topical application of poly(P) on gingival connective tissue and its remodeling in a rat periodontitis model.

Material and Methods: Male Wistar rats ($n = 36$, 8 wk of age) were used in this 6-wk study. The rats were divided into six groups of six rats each. The control group received no treatment. In the other groups, periodontitis was ligature-induced for 4 wk. After 4 wk, the rats with periodontitis were further divided into five groups, and were left untreated (periodontitis group) or subjected to topical application of oral rinses containing 0, 0.1, 1 or 5% poly(P) for 2 wk.

Results: The periodontitis and 0% poly(P) groups showed a higher density of polymorphonuclear leukocytes and a lower density of collagen in gingival tissue than the control group ($p < 0.05$). In contrast, groups treated with more than 1% poly(P) exhibited a lower density of polymorphonuclear leukocytes ($p < 0.05$) and a higher density of collagen than the periodontitis and 0% poly(P) groups ($p < 0.05$). A higher expression of fibroblast growth factor-2 was observed in the gingiva of rats treated with 1% poly(P) than in those treated with 0% poly(P) ($p < 0.05$).

Conclusion: Topical application of poly(P) may induce connective tissue remodeling, contributing to improvement of inflamed gingiva in rats.

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Periodontitis is a chronic inflammatory disease of the supporting tissues of teeth, which develops as a result of an imbalance between dental plaque biofilm and abnormal host responses to infection (1). Removal of dental plaque biofilm is the main aim of periodontal treatment. Studies have also shown that modulation of the host responses is important to allow spontaneous healing of inflamed periodontal tissues (2,3).

Inorganic polyphosphate [poly(P)], which is a linear polymer of many tens or hundreds of orthophosphate units, is found in almost all cells and tissues in nature (4). Poly(P) controls the activities of cell proliferation and collagen turnover in cultured human dental pulp cells (5). It also stabilizes fibroblast growth factor 2 (FGF-2) and increase the amount of FGF-2 that binds to FGF receptors (6), which is an important regulator of periodontal tissue remodeling (7). These histological changes induced by poly(P) may offer clinical benefits in the improvement of periodontitis.

MMPs, which are a family of structurally related zinc-dependent

endopeptidases, play an important role in a variety of biological processes, including tissue destruction (8,9) and also in tissue remodeling and wound healing (10,11). The activity of MMPs is regulated by natural inhibitors, particularly TIMPs, which inhibit MMP activity by binding the active site of enzymes (12). Regulating the balance between MMPs and TIMPs is essential to induce tissue remodeling and wound healing of gingival connective tissue. Therefore, poly(P) may contribute to the improvement of periodontitis by accelerating remodeling of the connective tissue through MMP/TIMP regulation. However, it is unclear how poly(P) affects gingival connective tissue and its remodeling in the periodontal lesion *in vivo*.

The purpose of the present study was to investigate the effects of topical application of poly(P) on gingival connective tissue and its remodeling in a rat periodontitis model. For better insight into the mechanism of action, we evaluated the histological changes, the expression of FGF-2, and the expression of *Mmp* and *Timp* genes.

Material and methods

Experimental design

All animal experiments complied with guidelines approved by the Animal Research Control Committee of Okayama University. Male Wistar rats (n = 36, 8 wk of age) were used in this 6-wk study. The rats were housed, two per cage, in rooms maintained at 23–25°C with 12-h light/dark cycles; the lights were off daily from 18.00 h to 06.00 h.

The rats were randomly placed into six groups of six rats each. One group (the control group) received no treatment for 6 wk. In the other groups, periodontitis was ligature-induced for 4 wk, after which the rats were left untreated (periodontitis group) or subjected to topical application of oral antibiotic rinses containing a cytotoxic agent (which destroys the cell membrane), either alone [0% poly(P) group] or supplemented with 0.1, 1 or 5% poly(P) (Regenetiss Inc., Tokyo, Japan), for 2 wk after removing the ligature. A 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a submarginal position of the maxillary second molars (13–15). Three successive applications of 5 μL of liquid containing poly(P) were administered once daily into the palatal gingival sulcus of both maxillary second molars.

Histological and immunohistochemical analysis

After the experimental period, the rats were killed under general anesthesia. The left maxillary molar region was resected *en bloc* from each rat. Tissues were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wk at 4°C. Paraffin-embedded buccolingual sections (4 μm thick) were stained with hematoxylin and eosin, or with other stains, as described below.

A commercial kit (Nichirei Co., Tokyo, Japan) was used to determine the level of FGF-2 expression. Polyclonal antibody against FGF-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted 1:200 in phosphate-buffered saline. The color was developed with 3-3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

A single, blinded examiner performed the following histometric analyses using a microscope at 200× or 400× magnification, after the preparations were assigned random numbers. The tissue sections stained with hematoxylin and eosin were used to evaluate histological changes. The linear distance between the cemento–enamel junction and the alveolar bone crest (an indicator of alveolar bone loss) was measured using a microgrid at a magnification of ×200 (16). The numbers of polymorphonuclear leukocytes (an indicator of inflammation), gingival fibroblasts, total cells and FGF-2-positive cells per unit area (0.1 mm × 0.1 mm) of the connective tissue subjacent to the junctional epithelium were determined under a magnification of ×400 (16,17).

The collagen density was determined in tissue sections stained with Mallory's aniline blue (18). Histological sections were analyzed under a standard microscope equipped with a digital camera. The color images generated by a camera were transferred to a microcomputer and analyzed using mathematical morphology software (WinROOF; Mitani Co., Fukui, Japan) (19). Measurements were performed in standard areas (0.05 mm × 0.05 mm each) subjacent to the junctional epithelium.

Isolation of RNA and determination of mRNA by RT-PCR

Total RNA was isolated from the gingival biopsy samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The isolated RNA was quantified by measuring the absorbance at 260 nm, and the purity was determined by the 260/280 nm absorbance ratio. Only samples with a ratio of > 1.8 were used (20). Total RNA (2 μg) was reverse transcribed using AMV Reverse Transcriptase (TaKaRa, Shiga, Japan) at 42°C for 30 min. The cDNA prepared as stated above was diluted 10-fold with yeast RNA (10 μg/mL). Real-time PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) in a LightCycler™ (Roche Applied Science, Mannheim, Germany) system, for 45 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The primer sequences of *Mmp2*, *Mmp3*, *Mmp9*, *Timp1*, *Timp2* and *Timp3* are shown in Table 1. Gene expression was calculated in terms of the relative copy number ratio of each mRNA to β-actin for each sample (expressed as mean ± SD).

Statistical analysis

The data were analyzed using a statistical software package (SPSS 17.0J for Windows; SPSS Japan, Tokyo, Japan). Parameters were analyzed statistically using one-way analysis of variance followed by Tukey's method (for comparisons among the three or seven groups). A p-value of < 0.05 was considered statistically significant.

Results

The periodontitis and 0% poly(P) groups showed a higher density of

Table 1. Primer sequences used for real-time PCR

Gene	Sense (5′–3′)	Antisense (5′–3′)	Amplicon/product length (bp)	Accession no.
Mmp2	TGGGGGAGATTCTCACTTTG	CCATCAGCGTTCCCATACTT	87	U65656
Mmp3	TGGGAAGCCAGTGGAAATG	CCATGCAATGGGTAGGATGAG	81	NM_133523
Mmp9	TGCTCCTGGCTCTAGGCTAC	TTGGAGGTTTTCAGGTCTCG	88	NM_031055
Timp1	CTGAGAAGGGCTACCAGAGC	GTCATCGAGACCCCAAGGTA	88	NM_053819
Timp2	AGGACCTGACAAGGACATCG	TTCTTTCCTCCAACGTCCAG	84	NM_021989
Timp3	TACACAGGGCTGTGCAACTT	CCAGGTGGTAGCGGTAATTG	85	U27201
Beta-actin	TGTTGCCCTAGACTTCGAGCA	GGACCCAGGAAGGAAGGCT	155	NM007393

polymorphonuclear leukocytes ($p < 0.05$) and a lower density of collagen ($p < 0.05$) than the control group (Table 2). Topical application of poly(P) induced a dose-dependent decrease in the number of polymorphonuclear leukocytes and a dose-dependent increase in the density of collagen. There were significant differences between the no-treatment group and groups treated with > 1% poly(P) ($p < 0.05$), and between the 0% poly(P) group and groups treated with > 1% poly(P) ($p < 0.05$) (Table 2 and Fig. 1). However, the groups treated with 1% and 5% poly(P) also showed a lower density of collagen than the control group. On the other hand, the distance between the cemento–enamel junction and the alveolar bone crest did not significantly differ among the groups receiving poly(P) treatment, and these values were significantly greater than the control group ($p < 0.05$).

FGF-2 staining was evident in the cytoplasm of fibroblasts, endothelial cells, epithelial cells and polymorphonuclear leukocytes (Fig. 2). The ratio of FGF-2-positive cells to total cells was higher in the gingiva treated with 0% and 1% poly(P) than that in the control group ($p < 0.05$) (Fig. 3). Furthermore, the gingiva treated with 1% poly(P) showed a higher ratio of FGF-2-positive cells to total cells than the 0% poly(P) group ($p < 0.05$). The density of gingival fibroblasts (mean ± SD) was 21.0 ± 2.3 in the 1% poly(P) group and 17.7 ± 1.3 in the 0% poly(P) group, and this difference was significant ($p < 0.05$).

The expression of *Timp2* and *Timp3* genes was more than twice as high in the 1% and 5% poly(P) groups than in the 0% poly(P) group ($p < 0.05$) (Table 3). The expression of the *Mmp9* gene was also more than twice as high in the 1% poly(P) group than in the 0% and 5% poly(P) groups ($p < 0.05$). On the other hand, there were no significant differences in expression of *Mmp2*, *Mmp3* and *Timp1* genes between the 0% and 1% poly(P) groups.

Discussion

In the present study, the periodontitis and 0% poly(P) groups showed periodontal inflammation, including polymorphonuclear leukocyte infiltration and collagen loss. However, the density of polymorphonuclear leukocytes within the connective tissue subjacent to the junctional epithelium was lower in the 1% and 5% poly(P) groups than in the periodontitis and 0% poly(P) groups. In addition, the collagen density in the gingival connective tissue was higher in the 1% and 5% poly(P) groups than in the periodontitis and 0% poly(P) groups. These observations indicate that topical application of poly(P) at a concentration of more than 1% could induce improvement in inflamed gingival connective tissue.

However, although studies have suggested that poly(P) induces regeneration of alveolar bone (5,21,22), the distance between the cemento–enamel junction and the alveolar bone crest did not change following the application of poly(P). In this study, poly(P) was applied to the gingival sulcus. Therefore, the local delivery of poly(P) may limit the effect of poly(P) on alveolar bone regeneration. In fact, a clinical study has demonstrated that bone regeneration by subgingival local irrigation with poly(P) was found in

Table 2. Histological findings in the periodontal tissue treated with different concentrations of inorganic polyphosphate [poly(P)]

			Concentration of poly(P)			
Parameter	Control	Periodontitis	0%	0.1%	1%	5%
Linear distance between the cemento–enamel junction and the alveolar bone crest	366 ± 50^{a}	664 ± 76^{b}	610 ± 87^{b}	636 ± 60^{b}	615 ± 101^{b}	635 ± 84^{b}
Density of polymorphonuclear leukocytes (0.1 mm × 0.1 mm)	1.1 ± 1.7^{a}	2.8 ± 0.7^{b}	2.6 ± 0.4^{b}	2.4 ± 0.6^{a,b}	0.9 ± 0.6^{a,c}	0.9 ± 0.4^{a,c}
Collagen density (%)	69.6 ± 5.5^{a}	42.5 ± 3.7^{b}	44.0 ± 4.3^{b,c}	43.9 ± 3.6^{b,c,d}	54.6 ± 6.0^{d}	54.0 ± 4.0^{d}

Data are given as mean ± SD. $n = 6$.

Mean values with the same superscript letter are not significantly different from one another, while those with different letters are significantly different (Tukey's test).

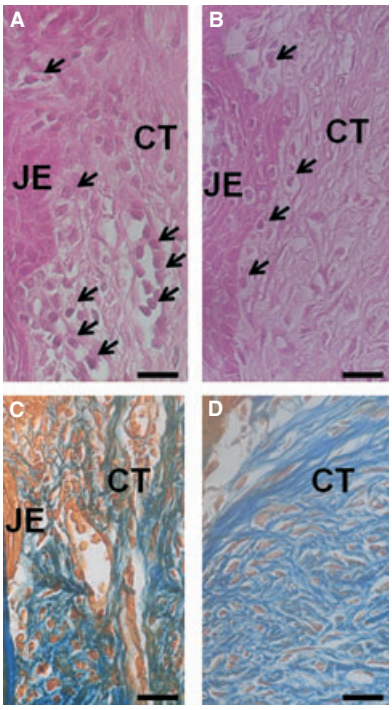

Fig. 1. Typical pattern of hematoxylin and eosin staining (A, B) and Mallory's aniline blue staining (C, D) in rat gingiva. Gingival connective tissue subjacent to the junctional epithelium receiving oral rinses containing 1% inorganic polyphosphate [poly(P)] (B, D) showed fewer polymorphonuclear leukocytes (arrows) and higher collagen density than the junctional epithelium receiving oral rinses containing 0% poly(P) (A, C), respectively. CT, connective tissue; JE, junctional epithelium. Scale bar = 100 μm.

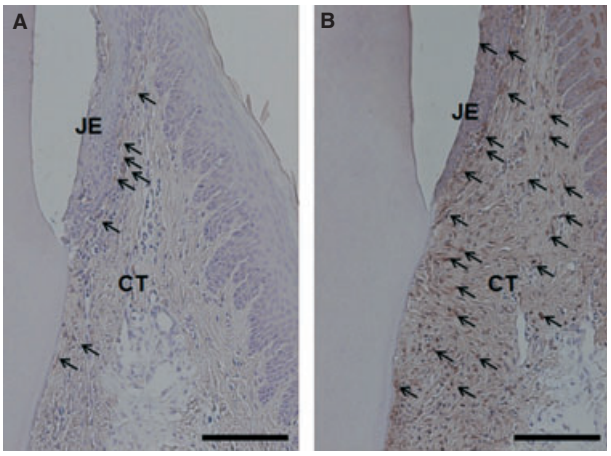

Fig. 2. Typical pattern of fibroblast growth factor 2 (FGF-2)-positive cells in rat gingiva. Gingivae receiving oral rinses containing 1% inorganic polyphosphate [poly(P)] (B) showed more FGF-2-positive cells (arrows) than the gingivae receiving oral rinses containing 0% poly(P) (A). CT, connective tissue; JE, junctional epithelium. Scale bar = 100 μm.

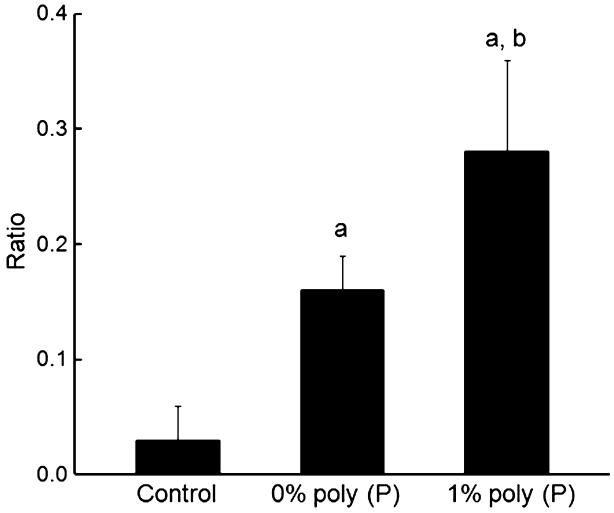

Fig. 3. Changes induced in the ratio of fibroblast growth factor 2 (FGF-2)-positive cells to total cells by inorganic polyphosphate [poly(P)]. [a]Differences compared with the control group were evaluated using the Tukey test ($p < 0.05$). [b]Differences compared with the 0% poly(P) group were evaluated using the Tukey test ($p < 0.05$).

only one of 16 patients with periodontitis (23).

In our findings, the 1% poly(P) group showed a higher ratio of FGF-2-positive cells to total cells than the 0% poly(P) group. The density of gingival fibroblasts was also higher in the 1% poly(P) group than in the 0% poly(P) group. These findings are consistent with a previous study revealing that poly(P) stabilized self-produced FGF-2, resulting in the enhancement of proliferation of cultured dental pulp cells (5). Many studies have shown that FGF-2 is a potent indicator of periodontal regeneration (7,24,25). It was also reported that proliferation of gingival fibroblasts is involved in periodontal tissue remodeling (17). Topical application of 1% poly(P) may con-

Table 3. Expression of *Mmp* and *Timp* genes in the 0%, 1% and 5% inorganic polyphosphate [poly(P)] groups

Gene	0% poly(P)	1% poly(P)	5% poly(P)
Mmp2	1.59 ± 0.56	1.24 ± 0.31	2.07 ± 0.06
Mmp3	1.39 ± 0.82	1.50 ± 0.21	0.59 ± 0.04
Mmp9	0.14 ± 0.14	0.72 ± 0.17[a]	0.22 ± 0.02[b]
Timp1	2.43 ± 1.62	2.73 ± 1.90	2.10 ± 0.10
Timp2	1.90 ± 0.08	4.90 ± 1.43[a]	4.84 ± 0.17[a]
Timp3	0.69 ± 0.32	1.61 ± 0.20[a]	1.62 ± 0.09[a]

Data are given as mean ± SD. $n = 3$–4.
[a]$p < 0.05$, compared with the 0% poly(P) group.
[b]$p < 0.05$, compared with the 1% poly(P) group.

tribute to gingival tissue remodeling through the enhancement of FGF-2 expression and gingival fibroblast proliferation.

The results also show that the 1% poly(P) group induced a higher level of expression of the *Mmp9* gene than the 0% poly(P) group. As FGF-2 induces the expression of *Mmp9* (26), this elevated mRNA level may have resulted from the enhancement of FGF-2 expression by poly(P). On the other hand, the 5% poly(P) group induced a lower level of expression of the *Mmp9* gene than the 1% poly(P) group. Although further studies are needed, negative feedback of *Mmp9* expression might increase according to the concentration of poly(P).

Furthermore, the expression of *Timp2* and *Timp3* genes were also upregulated by 1% and 5% poly(P). TIMP-2 inhibits MMP activity (12,27), while MMP-9 can degrade gingival connective tissue (28). Therefore, the induction of expression of *Mmp9* and *Timp2* genes in our findings suggests that poly(P) could accelerate the turnover of gingival connective tissue, and such conditions may result in the enhancement of collagen density. In addition, TIMP-3 seems to have an inhibitory effect on inflammatory cytokines, such as tumor necrosis factor-α (29). Poly(P) might also suppress periodontal inflammation through the upregulation of expression of the *Timp3* gene.

On the other hand, the 1% and 5% poly(P) groups exhibited a lower density of collagen than the control group. This suggests that the improvement of inflamed gingival tissue by poly(P) was incomplete. We had set an experimental period of 2 wk to apply poly(P). Longer periods of poly(P) application may be needed for complete improvement of the inflamed gingival connective tissue.

Ligature-induced periodontitis enhances bacterial dental plaque formation in rats (30). In this study, we applied poly(P) after removing the ligature. This indicates that improvement of inflamed gingival tissue by poly(P) application occurred when the substrate promoting dental plaque formation was removed. In other words, the present findings show that local delivery of poly(P) following the reduction of bacterial load helped to remodel the gingival connective tissue in periodontitis.

In our study, the bacteriostatic effect of oral rinses may also have an influence on the histological changes induced by poly(P). However, in our findings, there were no significant differences in histological changes between the periodontitis and the 0% poly(P) groups. This indicates that the bacteriostatic effect of oral rinses had little effect on the histological differences among the groups in which poly(P) was applied.

The use of therapeutic agents in dentifrices or oral rinses is a well-established approach for improving periodontal health. Poly(P) is a safe material with very low toxicity, and it is used in food additives and cosmetics. A previous study reported that supplementation of poly(P) in dentifrices is effective to suppress the formation of supragingival dental calculus (31). The present study revealed that poly(P), at a concentration of > 1%, is also an effective agent that improves inflamed gingival connective tissue. Supplementation of poly(P) in dentifrices and oral rinses would offer a beneficial option for prevention and healing of periodontal inflammation.

Our study had some limitations. For instance, we did not examine the effect of poly(P) on periodontal pathogens. Further studies are needed to clarify this issue. In addition, the ligature-induced rat periodontitis model is not directly equivalent to chronic disease in humans. It will be necessary to investigate how the local delivery of poly(P) affects gingival connective tissue in patients with chronic periodontitis.

In conclusion, topical application of poly(P) to the gingival sulcus induced FGF-2 expression, gingival fibroblast proliferation, and expression of *Mmp9*, *Timp2* and *Timp3* genes, contributing to the improvement of inflamed gingival connective tissue in rats.

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