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Effects of sub-antimicrobial dose doxycycline therapy on crevicular fluid MMP-8, and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis

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Objective: To investigate whether sub-antimicrobial dose doxycycline (SDD) therapy for 120 d in chronic adult periodontitis patients had significant effects on gingival crevicular fluid (GCF) matrix metalloproteinase-8 (MMP-8) levels, and on gingival tissue MMP-9, tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and interleukin-6 (IL-6) levels.

Background: Tetracycline can significantly inhibit MMP activity in GCF and in gingival tissue, even in much lower dosage then a traditional antimicrobial dosage used in conventional therapy. Sub-antimicrobial dose doxycycline (SDD) therapy has been shown to reduce periodontal disease activity to control MMP and pro-inflammatory cytokines.

Methods: A total of 32 patients with incipient to moderate (probing pocket depth $\approx$4–7 mm) chronic adult periodontitis were included in the study. Subjects were randomly assigned to two groups. After scaling and root planning (SRP), the SRP + SDD group received SDD, 20 mg bid, whereas the SRP + placebo group received placebo, 20 mg bid. In the follow-up, efficacy measures included the change in probing pocket depth (PD), clinical attachment level (CAL), bleeding on probing (BOP) and gingival crevicular fluid MMP-8 levels, gingival tissue MMP-9, TIMP-1 and IL-6 levels from baseline to 120 d.

Results: After 120 d, PD and CAL improved significantly in the SRP + SDD group. Initial MMP-8 levels for the SRP + SDD group and the SRP + placebo group were 407.13 ± 114.45 ng/ml and 378.71 ± 189.39 ng/ml, respectively, with no statistical difference between the two groups. MMP-8 levels for the SRP + SDD group and the SRP + placebo group were: 235.35 ± 134.58 ng/ml and 364.04 ± 219.27 ng/ml at 30 d; 157.50 ± 95.95 ng/ml and 236.60 ± 186.16 ng/ml at 60 d; 102.70 ± 67.64 ng/ml and 208.56 ± 124.54 ng/ml at 90 d; and 63.77 ± 53.33 ng/ml and 229.13 ± 168.09 ng/ml at 120 d, respectively. The amount of decrease in MMP-8 levels for the SRP + SDD group was statistically significant compared to that for the SRP + placebo group, especially apparent at 120 d ($p < 0.05$). TIMP-1 levels in both groups increased from the baseline to

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120 d with statistical significance (p-value < 0.05), but there was no significant difference between the two groups. Changes in MMP-9 and IL-6 levels were not statistically significant.

Conclusion: Adjunctive SDD therapy can improve the clinical parameters and this clinical improvement is reflected by controlled level of MMP-8 in chronic adult periodontitis after the therapy.

Tetracyclines have been used as an effective adjunct to periodontal therapy (1). Studies have shown that tetracyclines have a unique ability to be concentrated in gingival crevicular fluid (GCF) at a relatively higher level compared to serum level, and that they are effective in controlling the gram-negative organisms responsible for periodontal diseases (2). In addition to their use as systemically administered antibiotics, tetracyclines have been used as an effective root conditioning agent and a local delivery agent, largely due to their ability to bind to the tooth surface and to be released slowly (3).

More recent studies have shown that tetracycline, along with its closely related forms (doxycycline and minocycline), can be used in sub-antimicrobial dosage to control and suppress the progression of periodontal disease (4–6). These studies demonstrated that tetracycline families could significantly inhibit collagenase activity in GCF and in gingival tissue, even at a much lower dosage than a traditional antimicrobial dosage used in conventional periodontal therapy. Sub-antimicrobial dose doxycycline (SDD) therapy has been shown to reduce periodontal disease activity without inducing antimicrobial resistance (7).

Matrix metalloproteinases (MMPs) are enzymes involved in tissue destruction and regeneration. Extracellular matrix degradation during various periodontal disease is mediated by a complex cascade involving both host and microbial-derived proteinases (8). In this regards, the host-derived MMPs are thought to play a key role, and enhanced activity of these enzymes is a consequence of microbially induced inflammation in the periodontal tissues. Especially, polymorphonuclear leukocyte (PMN)-derived MMPs (MMP-8, MMP-9) are the main proteinases related to tissue destruction and remodeling events in periodontal diseases (9).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are modulating factors of MMPs activity, and four members of TIMPs have been reported. Among those, TIMP-1 and TIMP-2, which have inhibitory effects on all MMPs, are found in periodontal lesions (10). TIMP-2 shows strong inhibition against PMN-derived MMPs, whereas TIMP-1 shows greater inhibition against fibroblast-derived MMPs (11).

Interleukin (IL)-6 is a cytokine that is found at an increased level in GCF of periodontitis patients and is also reported to be closely related to clinical severity of periodontitis (12). Thus, studies on regulations of these factors are not only important to clarify pathogenic mechanisms but may also direct, at least in part, the therapeutic strategy.

The objective of the present study was to investigate whether SDD therapy for 120 d in chronic adult periodontitis patients had significant effects on GCF MMP-8 levels, and on gingival tissue MMP-9, TIMP-1 and IL-6 levels.

Materials and methods

Selection of human subjects

Patients with chronic incipient to moderate adult periodontitis treated in the Department of Periodontology, College of Dentistry, Yonsei University took part in the present study. Informed consent was obtained from all patients. The study was approved by the Review Board of Yonsei Dental Hospital, Seoul, Korea. A total of 32 adult human subjects between the ages of 25 and 64 years were randomly assigned to two groups as SRP + SDD group (SDD therapy following scaling and root planing) and SRP + placebo group (placebo following scaling and root planing).

The SRP SDD group consisted of 15 patients and the SRP + placebo group consisted of 17 patients. Patients in the SRP + SDD group ranged from 25 to 64 years of age with a mean age of 43, and patients in the SRP + placebo group ranged from 30 to 63 years of age with a mean age of 50. The SDD group consisted of eight males and seven females, and the control group consisted of nine males and eight females, with no statistical differences in either male/female ratio or age distribution between the two groups.

All subjects had no history of systemic disease or antibiotic therapy for 12 months prior to the experiment.

Clinical therapy and examination

The SRP + SDD group received scaling and root planing therapy and 20 mg bid SDD medication (Dentistar®, Hana Pharmaceutical Inc. Seoul, Korea), and the SRP + placebo group received root planing therapy and placebo (20 mg bid). One sextant was randomly selected from each patient, and the site with greatest initial pocket depth within the selected sextant was used as test site for the evaluation of clinical parameters. Scaling and root planing was done on one sextant per subject, and one specific tooth was chosen within the sextant for collecting gingival crevicular fluid and gingival tissue samples. Placebo and SDD therapy in the SRP + SDD and the SRP + placebo group was 120 d in duration. Changes in probing pocket depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) from baseline to 120 d were measured.

Gingival pocket depth measurement was made by one experienced clinician (D. H. Choi) using Marquis color-coded probe (diameter 0.5 mm) inserted with apical force of 20–30 *g*. Pocket depth was measured to the nearest 1 mm.

Sample collection

Fifteen patients received 20 mg doxycyline, and 17 patients received placebo tablets bid for 120 d. GCF samples were obtained from deep periodontal pockets (pocket depth $\approx$4–7 mm) before and on d 30, 60, 90 and 120 of drug intake. Prior to collection, the tooth surface was dried with air and kept dry with cotton wool rolls. Three paper points were inserted into the sulcus for 3 min, and then placed in a vial containing 200 μl of the enzyme reaction buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, pH 7.5). Adsorbed fluid was eluted from the paper points by vigorous vortexing the sample vial and centrifuged at 13,000 × *g* for 10 min at 4°C. Supernatants were collected and stored at −20°C until required.

Gingival tissue samples were obtained from patients before and on d 120 of drug intake. After surgery, excised tissue samples were immediately placed on ice and subsequently stored at −80°C. To prepare tissue extracts, samples were minced and homogenized in phosphate-buffered saline. After centrifuging to remove cell debris, the supernatants were collected and the protein concentration was determined using Coomassie-blue protein assay reagent (Pierce Chemical Co., Rockford, IL, USA).

Enzyme-linked immunosorbent assay (ELISA) for MMP-8, MMP-9, TIMP-1 and IL-6

MMP-8 levels in GCFs and levels of MMP-9, TIMP-1 and IL-6 in gingival tissue extracts were measured by using ELISA kits (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. ELISA kits for MMP-8, MMP-9, TIMP-1 and IL-6 had a linear binding curve from 0 to 4 ng/ml, from 0 to 16 ng/ml, from 0 to 400 pg/ml, and from 0 to 50 ng/ml, respectively.

Statistical analysis

All the data were presented as means ± standard deviation and results were statistically analyzed. Changes in MMP-8 levels from baseline values to each clinical stage (30, 60, 120 d) within each group and intergroup comparison were compared using ANOVA and *post hoc* test for multiple comparisons. Changes in MMP-9, TIMP-1 and IL-6 levels in gingival tissues from baseline values to the final values after 120 d within each group and intergroup comparison were compared using the same method.

Results

Patient distribution and clinical examination

A total of 32 patients with incipient to moderate level of chronic adult periodontitis entered the study. Pre-experiment pocket depth of the sites used in the study ranged from 4 to 7 mm, with mean pocket depth of 5.4 ± 1.2 mm for the SRP + SDD group and 5.5 ± 1.3 mm for the SRP + placebo group. There was no statistical difference in pre-experiment pocket depth between the two groups (Table 1). After 120 d, PD, CAL and BOP in both groups enhanced significantly compared to the baseline ($p < 0.05$).

Table 1. Clinical evaluations of the experimental sites at the baseline

Group	No.	Age (year)	Sex	Site	PD (mm)	BOP
SRP + SDD	1	33	M	35DL[a]	5	+
	2	39	M	32DL	4	+
	3	43	F	26DL	6	+
	4	34	F	37ML	6	+
	5	46	M	44MB	7	+
	6	45	F	47DB	7	+
	7	64	F	46ML	6	+
	8	46	M	15DB	4	+
	9	58	M	41MB	4	+
	10	43	F	35DL	6	+
	11	25	F	36DB	5	+
	12	30	M	36DB	7	+
	13	54	M	15ML	4	+
	14	35	M	25ML	6	+
	15	43	F	37DL	4	+
Mean ± SD		43 ± 10.56			5.4 ± 1.2	
SRP + placebo	1	54	M	25DL	6	+
	2	63	M	16DL	6	+
	3	58	F	16ML	6	+
	4	55	F	27DL	6	+
	5	58	F	46ML	4	+
	6	62	M	27DL	4	+
	7	39	M	47MB	6	+
	8	52	M	36ML	4	+
	9	63	F	26DB	6	+
	10	39	M	25MB	4	+
	11	46	F	25MB	6	+
	12	46	F	17DL	7	+
	13	55	M	17DL	7	+
	14	38	F	35DB	6	+
	15	45	M	15DL	6	+
	16	54	F	17DB	4	+
	17	30	M	16DB	6	+
Mean ± SD		50 ± 9.80			5.5 ± 1.3	

All values are expressed in mean ± SD.
M, male; F, female.
[a]Tooth type: MB, mesiobuccal; ML, mesiolingual; DB, distobuccal; DL, distolingual.
PD, initial probing depth; BOP, bleeding on probing; +, presence of BOP; –, absence of BOP.

Especially, statistical significant improvement in PD and CAL between baseline and after 120 d was observed in the SRP + SDD group (Table 2).

Evaluation of MMP-8 levels in GCF

MMP-8 levels in the GCF from the SRP + SDD group and the SRP + placebo group were measured every 30 d for 120 d (Fig. 1). Initial MMP-8 levels for the SRP + SDD group and the SRP + placebo group were 407.13 ± 114.45 ng/ml and 378.71 ± 189.39 ng/ml, respectively, with no statistical difference between the two groups. MMP-8 levels for the SRP + SDD group and the SRP + placebo group were: 235.35 ± 134.58 ng/ml and 364.04 ± 219.27 ng/ml at 30 d; 157.50 ± 95.95 ng/ml and 236.60 ± 186.16 ng/ml at 60 d; 102.70 ± 67.64 ng/ml and 208.56 ± 124.54 ng/ml at 90 d; and 63.77 ± 53.33 ng/ml and 229.13 ± 168.09 ng/ml at 120 d, respectively. MMP-8 levels in both groups gradually decreased as the experiment progressed for 120 d. In the SRP + SDD group, the decrease was statistically significant in every 30-d term compared to initial MMP-8 levels, whereas in the SRP + placebo group the difference was not statistically significant. The amount of decrease in MMP-8 levels for the SRP + SDD group was statistically significant compared to that for the SRP + placebo group, especially apparent at 120 d ($p < 0.05$).

Table 2. Changes of clinical parameters during 120 d

	Baseline			120 d		
Group	PD (mm)	CAL (mm)	BOP	PD (mm)	CAL (mm)	BOP
SRP + SDD	5.4 ± 1.2	6.4 ± 0.9	1.0 ± 0.0	3.8 ± 1.5*†	4.2 ± 1.5*†	0.3 ± 0.5*
SRP + placebo	5.5 ± 1.3	6.1 ± 1.1	1.0 ± 0.0	4.4 ± 1.5*	5.5 ± 1.2*	0.5 ± 0.5*

All values are expressed in mean ± SD.
PD, probing pocket depth (mm); CAL, clinical attachment level (mm); BOP, bleeding on probing.
*Statistically significant at $p < 0.05$ compared to baseline data.
†Statistically significant at $p < 0.05$ between SDD and placebo groups.

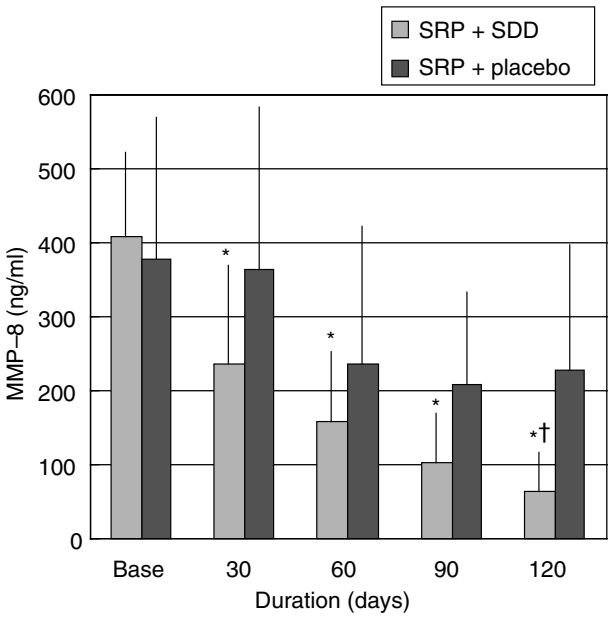

Fig. 1. The effect of sub-antimicrobial dose doxycycline or placebo administration on MMP-8 concentration in gingival crevicular fluid. *Statistically significant at $p < 0.05$ compared to baseline data. †Statistically significant at $p < 0.05$ compared to SRP + placebo group.

Changes in MMP-9 levels in gingival biopsy samples

In the SRP + SDD group, there were seven paired (initial and after 120 d) samples. In the SRP + placebo group, there were 11 paired samples. Initial MMP-9 levels for the SRP + SDD group and the SRP + placebo group were 11.19 ± 6.74 ng/mg protein and 12.97 ± 12.28 ng/mg protein, respectively, with no statistically significant difference. After 120 d, the SRP + SDD group showed a decreased MMP-9 level to 6.21 ± 3.92 ng/mg protein, whereas the SRP + placebo group showed an increase to 19.85 ± 19.15 ng/mg protein, but the differences were not statistically significant (Fig. 2). There was no statistically significant difference in MMP-9 levels between the SRP + SDD and the placebo group ($p < 0.05$).

Changes in TIMP-1 levels in gingival biopsy samples

Initial TIMP-1 levels for the SRP + SDD group and the SRP + placebo group were 7.10 ± 6.35 ng/mg, and 5.94 ± 4.72 ng/mg, respectively, with no statistically significant difference. After 120 d, TIMP-1 levels increased to 15.56 ± 4.33 ng/mg in the SRP + SDD group and to 12.78 ± 11.82 ng/mg in the SRP + placebo group (Fig. 3). In both groups, the increase in TIMP-1 levels from initial value to 120-d value was statistically significant ($p < 0.05$), but there was no significant difference between the two groups.

Changes in IL-6 levels in gingival biopsy samples

Initial IL-6 levels for SRP + SDD and SRP + placebo group were 15.92 ± 7.38 pg/mg, and 16.56 ± 8.67 pg/mg, respectively, with no statistically significant difference. After 120 d, the SRP + SDD group showed a decreased IL-6 level to 7.38 ± 4.86 pg/mg, and the SRP + placebo group also showed a slight decrease to 15.17 ± 9.09 pg/mg (Fig. 4). There was no statistically significant difference in IL-6 levels between the SRP + SDD group and the SRP + placebo group.

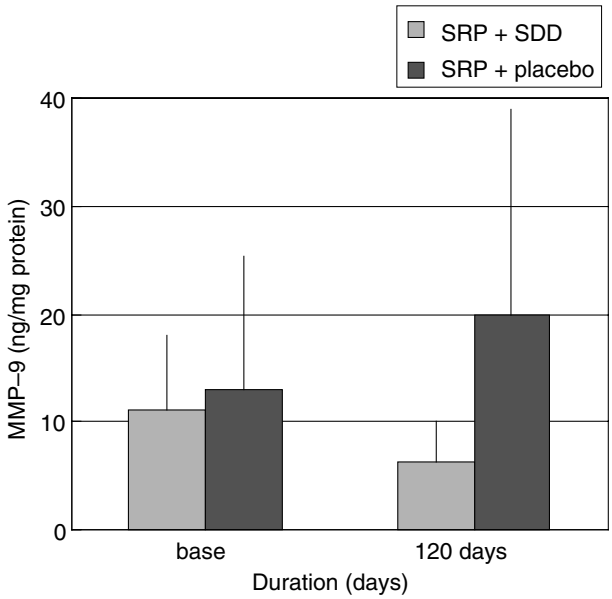

Fig. 2. The effect of sub-antimicrobial dose doxycycline or placebo administration on MMP-9 concentration in gingival biopsies.

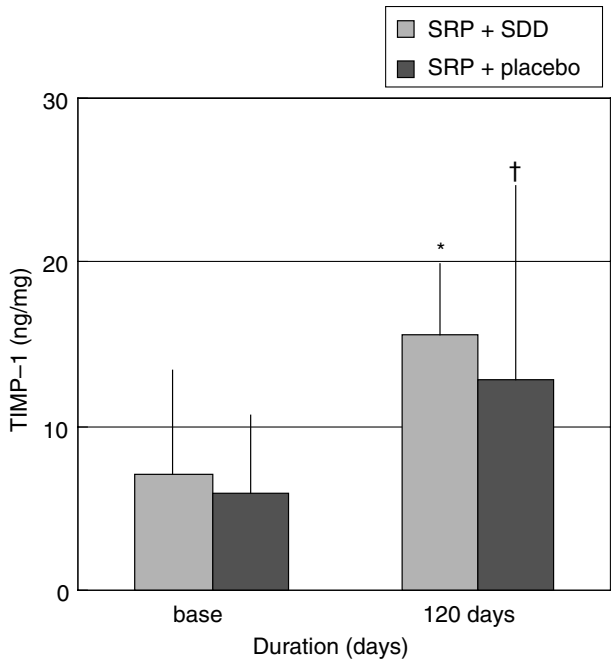

Fig. 3. The effect of sub-antimicrobial dose doxycycline or placebo administration on TIMP-1 concentration in gingival biosies. *Statistically significant at $p < 0.05$ compared to baseline data. †Statistically significant at $p < 0.05$ compared to baseline data.

Discussion

Doxycycline is one of the tetracycline derivatives that have been known for their effectiveness in antibiotic applications. Tetracyclines, due to their effectiveness in suppressing periodontopathic microorganisms (13), have been used widely as adjunctive periodontal therapeutic agents (14). Compared to other antibiotics, tetracyclines remain at a higher concentration in gingival crevicular fluid within the periodontal pockets. Later studies found non-antibiotic effects of tetracyclines which could further extend their clinical applications (15). Golub *et al.* suggested that SDD therapy could be used to control and suppress the progression of periodontal disease (4, 5). In addition to these results, Caton *et al.* reported that SDD therapy combined with scaling and root planing resulted in clinical improvement in chronic adult periodontitis patients (16). They also showed that this adjunctive SDD therapy did not have an antibiotic effect on periodontopathic microorganisms and caused no antibiotic resistance (17). These findings suggest that adjunctive SDD therapy can be used safely and effectively. This clinical effectiveness of SDD therapy, however, is not well-explained in terms of how SDD reduces periodontal inflammation clinically.

The present study is a double-blinded experiment designed to test the effectiveness of SDD as opposed to placebo in periodontally affected host tissue. This experiment design is used in order to rule out the effect of mechanical periodontal therapy and the psychological effect of both the patients and clinicians. After 120 d, PD, CAL and BOP of the experimental sites improved significantly. These improvements show the effect of mechanical therapy. Changes in PD and CAL, especially, were statistically significant compared to the SRP + placebo group, and these changes can be attributed to the effect of 120 d-long adjunctive SDD therapy.

We investigated whether 120 d of SDD therapy in chronic adult periodontitis patients had significant effects on GCF MMP-8 levels, and on gingival tissue MMP-9, TIMP-1 and IL-6 levels. These factors are thought to be the 'marker' of periodontal tissue breakdown in chronic adult periodontitis patients (18). MMPs are the main proteinases related to tissue destruction and remodeling events in periodontal diseases, and recent studies have shown the presence of neutrophil collagenase (MMP-8) and gelatinase (MMP-9) in inflamed human gingiva and GCF of adult periodontitis patients (19). Studies have shown that MMP-8 and MMP-9 were found at an increased level in GCF of chronic adult periodontitis patients (8). These MMPs

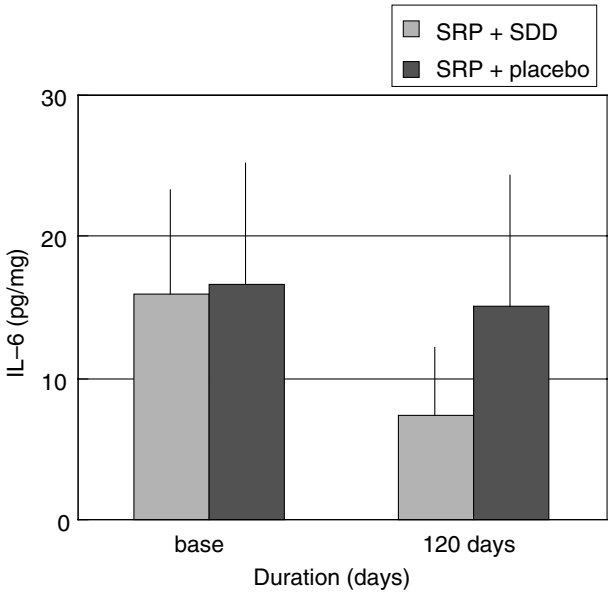

Fig. 4. The effect of sub-antimicrobial dose doxycycline or placebo administration on IL-6 concentration in gingival biopsies.

are PMN-derived factors, and based on findings that PMN is the origin of destructive enzymes in inflammatory periodontal tissue (20).

The results showed that the level of MMP-8 within the GCF samples of the SRP + SDD group decreased significantly as opposed to those from the SRP + placebo group. These findings are consistent with other studies (4, 5) that demonstrated that SDD therapy could significantly inhibit collagenase activity in gingival crevicular fluid and in gingival tissue. The differences between the two groups existed in all stage, but became apparent at 120 d of therapy, indicating that the duration of SDD therapy should be long-term in order to be effective.

But, the changes in MMP-9 levels were not statistically significant, even though the SRP + placebo group showed an increase in MMP-9 level, whereas the SRP + SDD group showed a decrease in MMP-9 level.

TIMP-1 is a modulating factor of MMP activity (21, 22). In order to test the effect of TIMP on fibroblast-derived MMPs (MMP-1, -2, -9), the present study evaluated changes in TIMP-1 levels. The results showed that TIMP-1 level increased in both SDD and placebo groups, with no statistically significant difference between the two groups. Increase in TIMP-1 level is probably due to a mechanical intervention that reduces the overall bacterial load in the oral cavity and to a reduction in MMPs which would bind to free TIMP (23–25). It is difficult to conclude the definite effect of SDD therapy on TIMP-1.

IL-6 is a cytokine that is also reported to be closely related to clinical severity of periodontitis. IL-6 is found at a high level in periodontal tissue of patients with clinically active periodontitis, and this elevated IL-6 level is shown to be reversed following successful periodontal therapy. In the present study, the SRP + SDD group showed a greater decrease in IL-6 level compared to the SRP + placebo group, although the difference was not statistically significant.

Although it is difficult to make definite conclusion on the positive effect of SDD therapy on MMP-9, TIMP-1, and IL-6, the effect of SDD on MMP-8 levels shown in this study can certainly support the clinical value of SDD therapy. Effects on other factors can be evaluated in future studies with larger number of samples, and further studies may be necessary to confirm these results.

In conclusion, adjunctive SDD therapy can improve the clinical parameters and this clinical improvement is reflected by controlled level of MMP-8 in chronic adult periodontitis after the therapy.

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