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# Adjunctive subantimicrobial dose doxycycline: effect on clinical parameters and gingival crevicular fluid transforming growth factor-$\beta_1$ levels in severe, generalized chronic periodontitis

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***Abstract***

**Background:** At present there is limited data concerning the efficacy of non-surgical periodontal therapy supplemented with subantimicrobial dose doxycycline (SDD) in the treatment of severe, generalized periodontitis. The purpose of the present study was to evaluate the effect of adjunctive SDD therapy on clinical periodontal parameters and gingival crevicular fluid (GCF) transforming growth factor-beta1 (TGF-$\beta_1$) levels in patients with severe, generalized chronic periodontitis over a 6-month period.

**Methods:** Thirty-five patients with severe, generalized periodontitis and 11 periodontally healthy subjects were included in the present study. Patients received full-mouth supragingival debridment at baseline and randomized to take either SDD b.i.d. or placebo b.i.d. for 3 months. Patients received root planing and oral hygiene instruction once a week for four consecutive weeks. Clinical measurements including probing depth (PD), clinical attachment level, papilla bleeding index and plaque index and GCF sampling were performed at baseline, 3 and 6 months. The GCF TGF-$\beta_1$ levels were analysed by enzyme-linked immunosorbent assay.

**Results:** Thirteen patients in both study groups completed the 6-month trial. Following scaling and root planing (SRP) plus SDD and SRP plus placebo therapy significant improvements in clinical periodontal parameters of both groups were observed ($p<0.025$). In the SDD group a significantly higher percentage (%73.4) of deep pockets resolved (PD reduction $\geqslant 3$ mm from baseline) when compared with placebo group (%49.7) at 6 months ($p<0.05$). At baseline there were no significant differences in GCF TGF-$\beta_1$ levels between three groups. Both total amount and concentration of GCF TGF-$\beta_1$ in SDD and placebo groups increased when compared with baseline at 3 months. However, only GCF TGF-$\beta_1$ levels of SDD group was significantly higher than baseline ($p<0.025$) and placebo group ($p<0.017$) at 3 months. At 6 months GCF TGF-$\beta_1$ levels of both groups were similar to baseline levels ($p<0.025$).

**Conclusions:** These data indicate that combination of SDD with non-surgical therapy improves clinical parameters of periodontal disease and increases GCF TGF-$\beta_1$ levels together with a decrease in prevalence of residual pockets in patients with severe, generalized chronic periodontitis. Increased GCF TGF-$\beta_1$ levels following SDD therapy might suggest a novell pleiotrophic mechanism for tetracyclines to inhibit connective tissue breakdown.

Key words: gingival crevicular fluid/analysis; matrix-metalloproteinases; periodontal disease/non-surgical therapy; subantimicrobial dose doxycycline; transforming growth factor-beta1.

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Production of host-derived enzymes, cytokines and other mediators by resident as well as inflammatory cells exaggerate immune and inflammatory reactions initiated in response to microbial factors and this leads to subsequent degradation of connective tissues during periodontal diseases (Okada & Murakami 1998, Kornman 1999). A large body of evidence suggests that connective tissue breakdown characteristic of chronic inflammatory diseases such as periodontitis are primarily mediated by matrix-metalloproteinases (MMPs) (Page 1991, Kinane 1992, Kinane 2000).

Transforming growth factor-beta1 (TGF-β1), a cytokine generally known for its potential effects on repair and regeneration, has important effects on regulation of MMPs. It inhibits the release of procollagenase and suppresses collagenase production by fibroblasts and macrophages (Edwards et al. 1987, Laiho & Keski-Oja 1989, Overall et al. 1989a, 1991, Page 1991). Moreover, it down-regulates transcription of MMP genes and enhances the expression of MMP inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor (Edwards et al. 1987, Overall et al. 1989a, b). TGF-$\beta_1$ is a key mediator not only in MMP regulation, but also in limitation and resolution of inflammation (Sodek & Overall 1992, Steinsvoll et al. 1999). This multifunctional cytokine has both pro-inflammatory and anti-inflammatory effects thus, it may be involved in both early inflammatory and in chronic destructive aspects of periodontal disease (Wahl et al. 1993). Suppression of both cell-mediated and humoral immune response is among the anti-inflammatory properties of TGF-$\beta_1$. Effects on cell proliferation and differentiation process, suggests a major role for this cytokine in wound healing, tissue remodeling and regeneration (Sporn & Roberts 1993). In addition, TGF-$\beta_1$ increases the synthesis of extracellular matrix molecules via stimulating numerous cell types (Chen et al. 1987, Fine & Goldstein 1987, Varga et al. 1987, Westergren-Thorsson et al. 1990, Matsuda et al. 1992, Hakkinen et al. 1996). TGF-$\beta_1$ has prominent effects in regulation of collagen matrix in physiological as well as in pathologic conditions like periodontitis (van der Zee et al. 1997). Expression and production of this cytokine not only at periodontally diseased but also at healthy sites suggests that it contributes to maintenance of tissue integrity (Okada & Murakami 1998, Steinsvoll et al. 1999, Buduneli et al. 2001, Wright et al. 2003). Therefore, these properties of TGF-$\beta_1$ indicate its apparent role in the pathogenesis of periodontal diseases and inflammatory wound healing.

Scaling and root planing (SRP) are effective treatment modalities in slowing or arresting periodontal diseases (Badersten et al. 1984). However, SRP does not directly target the host response component of periodontal disease. Therefore, novel therapeutic approaches have changed the trend towards pharmacologic modulation of exaggerated host response in addition to microbial elimination. Research on potential host-modulatory benefits of various pharmacological agents such as tetracyclines (TCs), non-steroidal anti-inflammatory drugs and bisphosphonates gave promising results (Golub et al. 1985, 1991, Greenwald et al. 1992, Howell 1993, Reddy et al. 1995, Buduneli et al. 2002). Extensive research on TCs and TC analogues has shown that this antibiotic family has non-antimicrobial properties that can modulate host-response by various mechanisms (Golub et al. 1991, 1992, 1998, Ingman et al. 1993). Current evidence suggests that TCs are able to inhibit collagenolytic activity through the inhibition of MMPs (Golub et al. 1985, 1987, 1990, 1994a). Suppression of MMP-mediated events with TCs results in decreased connective tissue breakdown and bone resorption (Golub et al. 1990, 1994b). It was shown that TCs sustained their MMP inhibitory effect even at doses below those needed for antimicrobial efficacy (Golub et al. 1990). In this regard, a specially formulated subantimicrobial dose doxycycline (SDD) was approved by Food and Drug Administration as an adjunct to non-surgical periodontal therapy (Caton 1999).

Previous double-blind, placebo-controlled studies on chronic periodontitis patients have reported that adjunctive SDD therapy yielded a significant reduction in probing depth (PD) and an increase in clinical attachment level (CAL) when compared with adjunctive placebo (Crout et al. 1996, Ciancio & Ashley 1998, Caton et al. 2000, Golub et al. 2001, Novak et al. 2002, Emingil et al. 2004). However, there are limited data regarding the clinical effect of non-surgical therapy supplemented with SDD in patients with a severe and generalized form of chronic periodontitis (Novak et al. 2002).

Previous studies evaluating host modulatory effects of TCs have elucidated that they have the ability to inhibit connective tissue breakdown by multiple mechanisms including enhancement of collagen production (Golub et al. 1998, Ryan & Golub 2000). The central role of TGF-$\beta_1$ in MMP modulation and collagen turnover as well as matrix deposition suggests that TCs might have potential effects on this cytokine. Therefore, in the present study we aimed to evaluate the effect of adjunctive SDD therapy on clinical periodontal parameters and GCF TGF-$\beta_1$ levels in patients with severe, generalized chronic periodontitis over a 6-month period.

## Materials and Methods

### Study population

Thirty-five male and female patients with severe, generalized chronic periodontitis were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir, Turkey. Medical and dental histories were taken and patients received clinical and radiographic evaluation at pre-screening visit. Inclusion criteria were being systemically healthy, having at least three natural teeth in each quadrant and at least a total of 14 teeth, diagnosed with severe, generalized chronic periodontitis (consensus report 1999), i.e. $>30\%$ sites with $\geqslant 5$ mm clinical attachment loss and having at least two sites with a PD $\geqslant 6$ mm in each quadrant that bled on probing. None of the patients had allergy or sensitivity to TCs and none had received antibiotics, non-steroidal anti-inflammatory drugs or periodontal therapy within 3 months of the pre-screening visit. Women who were pregnant, breast-feeding or using oral contraceptives were excluded. Eleven (six male and five female) systemically and periodontally healthy subjects ranged in age from 34 to 56 (mean age: $40.90 \pm 6.86$ years) were selected as controls. None of the subjects in the healthy group had clinical inflammation, bleeding on probing (BOP) or a history of medication use in the past 3 months. Patients who fulfilled the inclusion criteria provided written informed consent and participated in the study.

#### Study design

This was a double-blind, randomized, placebo-controlled, parallel group clinical trial of 6-month duration. Patients were randomized either to SDD or placebo groups. SDD group ($n = 17$; 13 male, four female) received SRP plus SDD capsules (containing doxycycline hyclate equivalent to 20 mg of doxycycline) and placebo group ($n = 18$; 13 male five female) was given SRP plus placebo capsules (containing inactive filler; i.e., cornstarch) b.i.d. for 3 months.

The design of the study is outlined in Fig. 1. Clinical trial was organized into five stages including pre-screening, screening, baseline, medication and evaluation. Pre-screening examination was conducted to evaluate patient eligibility for inclusion in the study. Patients eligible for the study returned to our clinic at screening visit for clinical measurements, 1 week after pre-screening. The periodontal status of each patient was assessed by a single examiner having experience in clinical trials. The full-mouth PD and CAL at six sites around each tooth were measured with a manual probe (Williams probe) using the cemento-enamel junction as the reference line. Third molar, partially erupted, endo-periodontally lesioned or crowned teeth and sites with subgingival restoration or caries and sites with restorations exceeding CEJ were excluded. Teeth with poor prognosis were extracted just before screening visit and sites adjacent to fresh extraction sockets were also excluded. The full-mouth papilla bleeding index (PBI) (Saxer & Muhlemann 1975) and plaque index (PI) (Quigley & Hein 1962) were also recorded. Clinical measurements were repeated at the last day of SDD or placebo therapy (3 months) and 3 months after the completion of the medication (6 months) by the same examiner who remained unaware to the treatment provided over the entire study period. Sites were subgrouped according to baseline PD into three groups: Group 1: shallow (0–3 mm), Group 2: mild-to-moderate (4–6 mm) and Group 3: deep ($\geqslant 7$ mm). The change in PD and CAL values of subgrouped sites from baseline and the percentage of deep pockets ($\geqslant 7$ mm at baseline) exhibiting PD reduction or increase more than 3 mm was assessed as efficacy measurements.

At baseline, 1 week after the screening visit, full mouth supragingival calculus removal was performed using an ultrasonic scaler. Following completion of the first session patients were randomized to SDD or placebo groups by the flip of a coin and were given 2-week doses of SDD or visually identical placebo capsules (28 capsules) in coded bottles. Patients were instructed to take capsules once in the morning and once in the evening at approximately 12 h intervals, 1 h before meals and to return unused capsules. In the following weekly sessions, root planing in each quadrant was performed with local anaesthesia by a single therapist blinded to the type of therapy with a time allowance up to 1 h per quadrant until smooth tooth surfaces were achieved. Root planing was conducted following a clockwise route starting from the upper right and ending at the lower right quadrant. Oral hygiene instruction including brushing, flossing and inter-dental brushing was given at baseline and controlled at each session. Patient compliance and any accompanying adverse effects were monitored at biweekly visits throughout the medication period. Patients who attended to these monitoring visits on exact day and ascertained to use all of the 28 capsules between two monitoring intervals via pill counts were assessed as ''compliant patients'' and were given additional 28 capsules. Monitoring of the adverse effects were continued by phone interviews every 4 weeks during the evaluation period. At recall visits following supragingival scaling, tooth surfaces were polished with a prophy paste and oral hygiene procedures were reinforced.

#### Gingival crevicular fluid (GCF) sampling

GCF sampling was done at baseline and was repeated at the last day of SDD or placebo therapy (3 months) and 3 months after the completion of the medication (6 months) before recording the clinical parameters. GCF samples of chronic periodontitis patients were collected from mesiobuccal aspect of a single-rooted tooth in upper right quadrant exhibiting PD of 6–8 mm. In healthy group GCF samples were collected from mesiobuccal aspect of a single-rooted tooth in upper right quadrant exhibiting PD up to 3 mm without inflammation and BOP. Prior to GCF sampling, the supragingival plaque was removed from the sampling sites with a sterile curette, these surfaces were dried

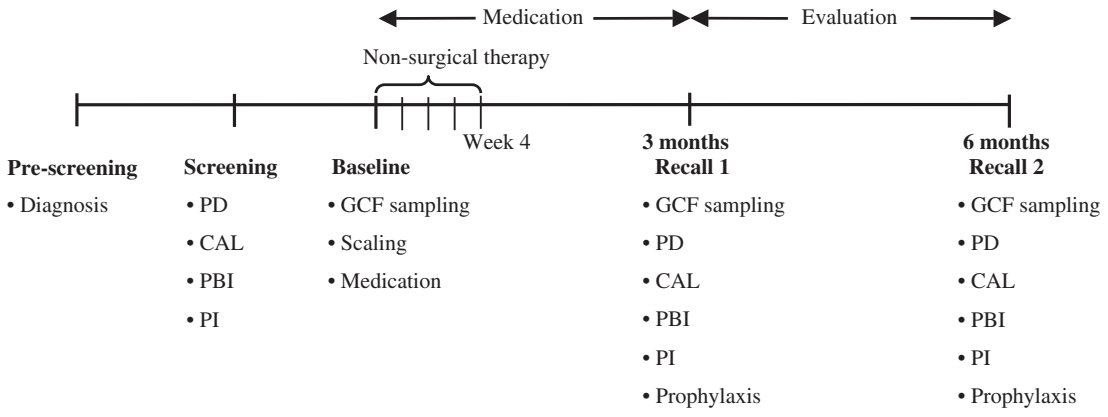


*Fig. 1.* Study design: following pre-screening and screening visits non-surgical therapy started at baseline with full-mouth supragingival scaling and followed by 4 weekly sessions of quadrant root planing and oral hygiene instruction. Adjunctive SDD or placebo administered for 3 months starting at baseline visit. Recall visits at 3 months (recall 1) and 6 months (recall 2) consisted of GCF sampling, clinical measurements, prophylaxis and reinforcement of oral hygiene procedures. PD, probing depth; CAL, clinical attachment level; PBI, papilla bleeding index; PI, plaque index; GCF, gingival crevicular fluid.

gently by an air syringe and were isolated by cotton rolls. GCF was sampled with filter paper (Periopaper, ProFlow, Inc., Amityville, NY, USA). Paper strips were non-traumatically inserted into the crevice until mild resistance was felt and left there for 30 s. Strips contaminated with blood were discarded. The absorbed GCF volume of each strip was determined by an electronic device (Periotron 8000, Proflow, Inc.) and placed into a sterile polypropylene tube and kept at $-40^{\circ}$C until being analysed. The readings from the Periotron 8000 (ProFlow, Inc.).were converted to an actual volume (μl) by reference to the calibration curve.

#### GCF TGF-$\beta_1$ analysis

GCF TGF-$\beta_1$ levels were analysed by enzyme linked immunosorbent assay (ELISA). GCF samples were eluted from the strips by placing them in 200 μl of phosphate-buffered solution. In order to measure biological active TGF-$\beta_1$, GCF samples were acidified using 20 μl 1 N HCl at room temperature for 15 min according to the manufacturer's instructions. Then, the samples were neutralized by adding 20 μl 1 N NaOH. The amount of TGF-$\beta_1$ was determined by using the relevant ELISA kit (Bendermed Systems Diagnostics, Vienna, Austria). The minimum detectable concentration of TGF-$\beta_1$ was 1.9 pg/ml. Results were received as total TGF-$\beta_1$ (pg/sample) in the GCF sample. Calculation of the TGF-$\beta_1$ concentration in each GCF sample was performed by dividing the amount of TGF-$\beta_1$ by the GCF volume of the sample (TGF-$\beta_1$ concentration pg/μl = total TGF-$\beta_1$ pg/GCF volume μl).

#### Statistical analysis

Statistical analysis was performed on data obtained from patients who completed the 6-month trial. The mean per-patient values of whole mouth PBI and PI and PD, CAL, PBI, PI measurements and GCF TGF-$\beta_1$ levels of sampling sites for both groups were calculated. Per-patient PD and CAL values of subgrouped sites and percentage of sites that exhibited PD change $\geqslant$3 mm from baseline with an initial PD $\geqslant$7 mm were also averaged at each time point.

Demographic variables were analysed using two-tailed Fisher's exact test. Mann–Whitney test was used to detect significant differences between groups for baseline clinical periodontal parameters of sampling sites, baseline PD and CAL values of subgrouped sites and percentage of deep pockets that attained thresholds. Intra-group comparisons of clinical periodontal parameters of sampling sites, full mouth PBI and PI and PD and CAL values of subgrouped sites were assesed by Friedman test followed by Bonferroni corrected Wilcoxon signed ranks test to analyse significant changes over time. $P$ values $<0.025$ were considered statistically significant. The Mann–Whitney test with a significance level $p<0.05$ was used to determine significant differences between the SDD and placebo groups.

Intra-group comparisons of GCF volume and GCF TGF-$\beta_1$ levels of SDD and placebo groups were also carried out using Friedman test. When there were significant differences intra-group comparisons were performed by using Wilcoxon signed ranks test. $P$ values $<0.025$ were considered statistically significant. Inter-group comparisons of GCF volume and GCF TGF-$\beta_1$ levels of healthy, SDD and placebo groups were performed using Kruskall–Wallis test followed by Bonferroni corrected Mann–Whitney test. $P$ values $<0.017$ were considered statistically significant.

## Results

#### Patient disposition and demographics

Of the 35 patients enrolled in the study, two patients withdrew because of social reasons within the first 4 weeks and seven patients failed to comply with the study protocol (i.e. drug regimen and monitoring schedule) were excluded from the study during the medication period. Random assignment resulted in 13 patients in the SDD group and 13 patients in the placebo group. None of the 35 patients complained of any adverse effects from the use of either SDD or placebo capsules.

Patient demographics for patients completing the 6-month follow up are shown in Table 1. The sex, mean ages and smoking habits of patients were similar in both groups. There were no ex-smokers in both SDD and placebo groups.

#### Whole mouth clinical findings

A total of 3422 sites were evaluated in 26 patients. Distribution of sites according to baseline PD by SDD and placebo groups is outlined in Table 2. Shallow, mild to moderate and deep sites were distributed identically between groups at baseline ($p>0.05$).

At baseline, there were no statistically significant differences in PD values of sites subgrouped by baseline PD between study groups ($p>0.05$) (Table 3). No statistically significant changes were observed at sites with a baseline PD 0–3 mm in both groups ($p>0.05$) (data not shown). Significant PD reductions were observed at sites with a baseline PD 4–6 mm and $\geqslant$7 mm as would be predicted and this reduction was maintained over the entire study period ($p<0.025$). Although the mean PD reduction for sites with a baseline PD 4–6 mm and $\geqslant$7 mm were greater in SRP plus SDD group than SRP plus placebo group at 6 months (1.80 mm *versus* 1.46 mm for mild-to-moderate pockets; 3.38 mm *versus* 2.57 mm for deep pockets) the differences did not reach to significance ($p>0.05$) (Table 3). Analysis of sites with a baseline PD $\geqslant$7 mm revealed that higher percentage of sites were reduced by at least 3 mm following adjunctive SDD therapy

*Table 1*. Demographic characteristics and smoking habits of patients completing the 6-month trial

| | SDD group | Placebo group |
|---|---|---|
| *n* | 13 | 13 |
| Age (mean years ± SD) | 46.38 ± 7.63 | 46.77 ± 6.95 |
| Age range | 34–59 | 35–59 |
| Male: female | 10:3 | 9:4 |
| Smokers (*n*) | 4 | 5 |

SDD, sub-antimicrobial dose doxycycline.

*Table 2*. Distribution of sites subgrouped by baseline probing depth in SDD and placebo groups

| Baseline PD | SDD group | Placebo group | Total |
|---|---|---|---|
| 0–3 mm | 638 (36.21%) | 660 (%39.76) | 1298 |
| 4–6 mm | 748 (42.45%) | 686 (%41.32) | 1434 |
| $\geqslant$7 mm | 376 (21.34%) | 314 (%18.92) | 690 |
| Total | 1762 | 1660 | 3422 |

SDD, sub-antimicrobial dose doxycycline; PD, probing depth.

(66.4%) than following adjunctive placebo therapy (55.1%) at 3 months, with no significant differences detectable between groups ($p > 0.05$). However, at 6 months percentage of sites that exhibited improved PD by $\geqslant 3$ mm were significantly higher ($p = 0.011$) in adjunctive SDD group (73.4%) than adjunctive placebo group (49.7%) (Fig. 2). None of the deep pockets exhibited a PD increase more than or equal to 3 mm during the study period.

At baseline, there were no statistically significant differences in CAL values of sites subgrouped by baseline PD between both study groups ($p > 0.05$) (Table 3). Sites with mild-to-moderate and deep pockets initially, exhibited significant CAL improvements at 3 and 6 months, when compared with baseline ($p < 0.025$). Sites with an initial PD 0–3 mm did not exhibit statistical significant CAL chances over the study period ($P > 0.05$). As an expected result of SRP therapy, sites with a baseline PD 0–3 mm exhibited slight loss of attachment with adjunctive placebo therapy ($-0.04$ mm at 3 months, $-0.03$ mm at 6 months). On the other hand, SRP plus SDD therapy demonstrated a slight attachment gain at sites with a baseline PD 0–3 mm (0.11 mm at 3 months, 0.14 mm at 6 months), but no significant difference regarding CAL changes was detected between study groups ($p > 0.05$). Although the mean CAL gain for sites with a baseline PD 4–6 mm and $\geqslant 7$ mm were greater in SRP plus SDD group when compared wih SRP plus placebo group at 6 months (1.12 mm *versus* 0.78 mm for mild to moderate pockets; 2.15 mm *versus* 1.76 mm for deep pockets) statistical analysis did not reveal significance ($p > 0.05$) (Table 3).

The full-mouth PBI and PI scores of both SDD and placebo groups markedly improved between baseline and the re-examinations at 3 and 6 months ($p < 0.025$). The reduction in PBI and PI was similar for both groups at all time points ($p > 0.05$) (data not shown).

#### Clinical findings of sampling sites

At baseline no significant differences were detected between SDD and placebo groups regarding the PD, CAL, PBI and PI of sampling sites. ($p > 0.05$) The clinical parameters of GCF sampling sites markedly improved during the 6-month study period ($p < 0.025$). Mean PD, CAL and PI values of sampling sites showed similar improvements between study groups at all time points ($p > 0.05$). In contrast, SRP plus SDD therapy resulted in statistically significant reduction in PBI over the SRP plus placebo therapy at 6 months ($p < 0.05$) (Table 4). GCF volume of sampling sites significantly improved during the 6-month study period ($p < 0.025$). GCF volume of both SDD and placebo groups were found to be statistically higher than that of healthy group at all time intervals ($p < 0.017$) (Fig. 3).

#### Laboratory findings of sampling sites

Although Kruskall–Walis test revealed significant difference ($p = 0.032$) in GCF TGF-$\beta_1$ total amount of SDD, placebo and healthy groups at baseline, Bonferroni corrected Mann–Whitney test failed to detect significant difference between SDD and placebo ($p = 0.028$), SDD and healthy ($p = 0.023$) or placebo and healthy ($p = 0.928$) groups ($p > 0.017$). At 3 months GCF TGF-$\beta_1$ total amount of SDD group significantly increased when compared with baseline ($P < 0.025$). At 6 months GCF TGF-$\beta_1$ total amount of SDD group was found to be similar to baseline levels

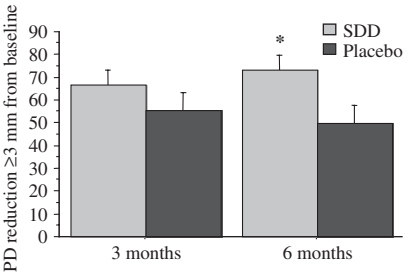


*Fig. 2.* Percentage of deep pockets ($\geqslant 7$ mm probing depth (PD) at baseline) that exhibited PD reduction $\geqslant 3$ mm from baseline. *Significant difference from placebo group ($p < 0.05$).

*Table 3.* The per-patient mean PD and CAL levels of diseased sites (4–6 mm and $\geqslant 7$ mm at baseline) in SDD and placebo groups (mean ± SE)

| | SDD group | | | Placebo group | | |
|---|---|---|---|---|---|---|
| | baseline | 3 months | 6 months | baseline | 3 months | 6 months |
| PD 4–6 mm | 4.97 ± 0.08 | 3.23 ± 0.13* | 3.17 ± 0.13* | 4.97 ± 0.07 | 3.44 ± 0.10* | 3.51 ± 0.15* |
| CAL 4–6 mm | 6.16 ± 0.18 | 5.17 ± 0.17* | 5.04 ± 0.17* | 6.11 ± 0.30 | 5.10 ± 0.27* | 5.33 ± 0.32* |
| PD $\geqslant 7$ mm | 7.67 ± 0.10 | 4.45 ± 0.30* | 4.29 ± 0.26* | 7.43 ± 0.08 | 4.65 ± 0.15* | 4.86 ± 0.25* |
| CAL $\geqslant 7$ mm | 8.63 ± 0.29 | 6.79 ± 0.30* | 6.48 ± 0.28* | 8.12 ± 0.21 | 6.38 ± 0.39* | 6.36 ± 0.40* |

*Significant difference from baseline ($p < 0.025$).
SDD, sub-antimicrobial dose doxycycline; PD, probing depth; CAL, clinical attachment level.

*Table 4.* Clinical parameters of sampling sites in SDD and placebo groups (mean ± SE)

| | SDD Group | | | Placebo group | | |
|---|---|---|---|---|---|---|
| | baseline | 3 months | 6 months | baseline | 3 months | 6 months |
| PD (mm) | 7.39 ± 0.15 | 3.77 ± 0.28* | 3.46 ± 0.24* | 7.31 ± 0.24 | 3.85 ± 0.22* | 4.00 ± 0.23* |
| CAL (mm) | 8.70 ± 0.55 | 6.77 ± 0.64* | 6.31 ± 0.60* | 8.39 ± 0.55 | 6.39 ± 0.53* | 6.54 ± 0.45* |
| PBI | 3.39 ± 0.27 | 0.46 ± 0.14* | 0.39 ± 0.10*† | 3.31 ± 0.24 | 0.31 ± 0.13* | 0.85 ± 0.20* |
| PI | 3.62 ± 0.24 | 1.76 ± 0.20* | 1.39 ± 0.18* | 3.54 ± 0.35 | 1.62 ± 0.14* | 1.62 ± 0.18* |

*Significant difference from baseline ($p < 0.025$).
†Significant difference from placebo group ($p < 0.05$).
SDD, sub-antimicrobial dose doxycycline; PD, probing depth; CAL, clinical attachment level; PBI, papilla bleeding index; PI, plaque index.

($p > 0.025$). GCF TGF-$\beta_1$ total amount of SDD group was significantly higher than the amount of healthy group at 3 months ($p < 0.017$). No significant differences in GCF TGF-$\beta_1$ total amount were found between SDD and healthy groups at 6 months ($p > 0.017$). GCF TGF-$\beta_1$ total amount of placebo group was similar to baseline levels at 3 months and 6 months ($p > 0.025$). This group had also similar GCF TGF-$\beta_1$ total amount to healthy group at both 3 months and 6 months ($p > 0.025$). At 3 months, GCF TGF-$\beta_1$ total amount of SDD group was significantly higher than the amount of placebo group ($p < 0.017$). However, at 6 months GCF TGF-$\beta_1$ total amount of both groups were similar ($p > 0.017$) (Table 5, Fig. 4).

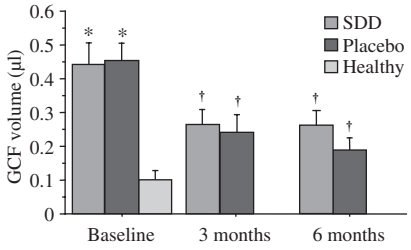


*Fig. 3.* Gingival crevicular fluid volume (µl) of sampling sites in SDD, placebo and healthy groups. Each value represents mean values and standard errors. *Significant difference from healthy group ($p < 0.017$). †Significant difference from baseline ($p < 0.025$) and healthy group ($p < 0.017$).

GCF TGF-$\beta_1$ concentration of SDD, placebo and healthy groups were found to be similar at baseline ($p = 0.244$), although the mean GCF TGF-$\beta_1$ concentration of healthy group seemed to be markedly higher than GCF TGF-$\beta_1$ concentration of both SDD and placebo groups. GCF TGF-$\beta_1$ concentration of SDD and placebo groups showed an increase following adjunctive therapy at 3 months, however only GCF TGF-$\beta_1$ concentration of SDD group showed significant improvement when compared with baseline ($p < 0.025$). At 3 months SRP plus SDD therapy revealed a significant increase in GCF TGF-$\beta_1$ concentration over the placebo group ($p < 0.017$). GCF TGF-$\beta_1$ concentrations of both groups were found to be similar to baseline levels ($p > 0.025$) at 6 months, although GCF TGF-$\beta_1$ concentrations of both groups were higher than baseline levels ($p > 0.025$) (Table 5, Fig. 5).

## Discussion

The present double-blind, placebo-controlled, parallel group study was designed to evaluate the efficacy of SRP plus SSD therapy in severe, generalized chronic periodontitis patients. Improvement of both clinical parameters and GCF TGF-$\beta_1$ levels observed in response to SDD therapy beyond that attained by non-surgical periodontal therapy alone could be tested.

It is well known that the efficacy of non-surgical therapy at an individual site is related to the baseline PD and deeper pockets have more potential of PD reduction and CAL gain (Cobb 1996). Therefore, we stratified sites by baseline PD and the effectiveness of non-surgical therapy was evaluated separately for these sites. The severity of chronic periodontitis was similar in both groups as indicated by baseline clinical parameters. SRP therapy leads to the resolution of the inflammatory response and arrests the progression of periodontal disease, resulting in clinical attachment gain and PD reduction (Greenstein 1992, Cobb 1996). As one might expect, significant improvements in clinical parameters were observed following both SRP plus SDD and SRP plus placebo therapy and these improvements were maintained throughout the study.

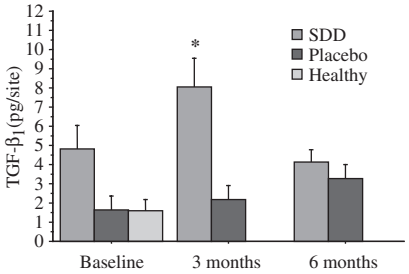


*Fig. 4.* Gingival crevicular fluid TGF-$\beta_1$ total amount (pg/site) of sampling sites in SDD, placebo and healthy groups. Each value represents mean values and standard errors. *Significant difference from baseline ($p < 0.025$), healthy and placebo group ($p < 0.017$).

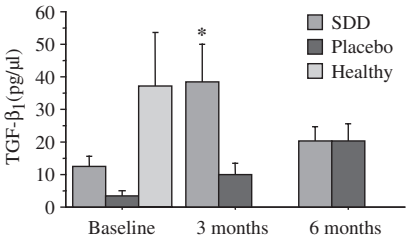


*Fig. 5.* Gingival crevicular fluid TGF-$\beta_1$ concentration (pg/µl) of sampling sites in SDD, placebo and healthy groups. Each value represents mean values and standard errors. *Significant difference from baseline ($p < 0.025$) and placebo group ($p < 0.017$).

*Table 5.* GCF TGF-$\beta_1$ total amount and concentration

| | | TGF-$\beta_1$ total amount (pg/site) | | | TGF-$\beta_1$ concentration (pg/µl) | | |
|---|---|---|---|---|---|---|---|
| | | Baseline | 3 months | 6 months | Baseline | 3 months | 6 months |
| SDD | Mean | 4.80 | 8.05* | 4.12 | 12.51 | 38.60† | 20.22 |
| | SD | 4.56 | 5.44 | 2.43 | 10.98 | 41.06 | 16.65 |
| | SE | 1.27 | 1.51 | 0.67 | 3.05 | 11.39 | 4.62 |
| | Median | 4.08 | 7.92 | 5.76 | 12.20 | 27.67 | 18 |
| | Minimum | 0 | 0.96 | 0 | 0 | 9.45 | 0 |
| | Maximum | 16.56 | 19.20 | 6.48 | 31.38 | 161.68 | 46.22 |
| Placebo | Mean | 1.63 | 2.20 | 3.29 | 3.54 | 10.12 | 20.27 |
| | SD | 2.71 | 2.52 | 2.50 | 4.85 | 12.14 | 19.06 |
| | SE | 0.75 | 0.70 | 0.69 | 1.35 | 3.37 | 5.29 |
| | Median | 0.72 | 1.44 | 3.6 | 1.50 | 5.23 | 14.34 |
| | Minimum | 0 | 0 | 0 | 0 | 0 | 0 |
| | Maximum | 10.08 | 7.20 | 7.68 | 17.08 | 38.92 | 56.89 |
| | | | Baseline | | | Baseline | |
| Healthy | Mean | | 1.59 | | | 37.16 | |
| | SD | | 1.97 | | | 54.13 | |
| | SE | | 0.59 | | | 16.32 | |
| | Median | | 0 | | | 0 | |
| | Minimum | | 0 | | | 0 | |
| | Maximum | | 5.04 | | | 163.20 | |

*Significant difference from baseline ($p < 0.025$), healthy and placebo groups ($p < 0.017$).
†Significant difference from baseline ($p < 0.025$) and placebo groups ($p < 0.017$).
SDD, sub-antimicrobial dose doxycycline; TGF, transforming growth factor; GCF, gingival crevicular fluid; SD, standard deviation; SE, standard error.

Previous double-blind, placebo-controlled studies evaluating the efficacy of adjunctive SDD therapy in chronic periodontitis patients have revealed additive effect of this treatment modality on mean PD reduction and CAL gain when compared with SRP plus placebo (Crout et al. 1996, Ciancio & Ashley 1998, Caton et al. 2000, Golub et al. 2001, Novak et al. 2002, Emingil et al. 2004). The findings of our study are in accordance with the findings previously reported. Although the mean inter-treatment differences at month 6 regarding PD reduction and CAL gain of diseased sites were higher than that was reported in previous large-scale trials (Ciancio & Ashley 1998, Caton et al. 2000, Golub et al. 2001), statistical analysis did not reveal significance. This might be the result of relatively small number of patients completing the present study.

Two-week dosing regimen and visit process repeated during the medication period enabled monitoring of patient compliance and possible adverse effects in an efficient manner. Of the 35 patients enrolled in the present study seven patients (i.e. 20%) were excluded. This may seem a high rate of exclusion for a study of 6-month duration and one may consider whether these exclusions are because of adverse events. In the present study the sole reason of exclusion was lack of compliance. This might probably result from the to some extent strict criteria in determining compliant patients. In the present study, 3 months of SDD therapy was well tolerated and none of the compliant or non-compliant patients reported adverse effects including gastrointestinal disturbances. These findings likely support the idea that SDD as an adjunct to non-surgical periodontal therapy is a safe therapautic approach in long term management of chronic periodontitis.

Caton et al. (2000) have proposed that determining per-patient mean values may be of less clinical significance than determining sites that exhibit improvements exceeding threshold levels. They have stated that PD reductions 3 mm or more might represent marked improvements of individual sites and might be of clinical significance. In the present study, when the percentage of severely diseased sites i.e. PD $\geqslant$7 mm exhibiting PD reduction at least 3 mm from baseline taken into consideration significantly greater percentage of sites (24% more sites than that of placebo) receiving adjunctive SDD was observed to attain this threshold level of PD reduction than sites receiving adjunctive placebo at 6 months. This result could be of particular importance since sites with a baseline PD $\geqslant$7 mm are strong candidates for surgical procedures. Therefore, it could be hypothesed that adjunctive SDD therapy might reduce the probability and discomfort of surgical intervention and repeated non-surgical therapy or application of local or systemic chemotherapeutics. It was stated that adjunctive SDD therapy prevented attachment loss at shallow sites (Ciancio & Ashley 1998). We have also demonstrated that shallow sites gained slight attachment following adjunctive SDD when shallow sites in placebo group experienced a slight attachment loss. These findings overall suggest that clinical improvements observed in SDD group might not be solely attributed to the efficacy of SRP, but could also be because of long-term host-modulatory benefits of SDD therapy (Golub et al. 1991, 1992, 1998, Ingman et al. 1993).

TGF-$\beta_1$ levels have been shown to be higher in gingival tissues and GCF at sites of inflammation when compared with healthy sites (Buduneli et al. 2001, Wright et al. 2003). In the present study we have demonstrated that all groups had similar GCF TGF-$\beta_1$ total amount at baseline. Considering anti-inflammatory role of TGF-$\beta_1$, markedly high levels of this cytokine might be expected since sampling sites were severely diseased and inflamed. Therefore, it could be hypothesed that GCF samples from study groups were collected from active disease sites where there is an imbalance between anti-inflammatory and destructive factors (Steinsvoll et al. 1999). However, when the data were expressed as concentration healthy group had higher GCF TGF-$\beta_1$ levels than both SDD and placebo groups. Skaleric et al. (1997) have demonstrated that GCF TGF-$\beta_1$ concentration positively correlated with PD. On the other hand, in ligature-induced animal periodontitis part of the same study, the authors detected a sharp decline in TGF-$\beta_1$ concentration with the increased PD. It is difficult to compare severely diseased human and animal conditions since the literature lacked of a study determining TGF-$\beta_1$ levels in GCF samples from patients with severe periodontitis. Our GCF TGF-$\beta_1$ concentration findings are in agreement with a previous report, which detected lower GCF TGF-$\beta_1$ concentration at inflamed sites when compared with healthy sites (Buduneli et al. 2001). Expression of GCF constituents as concentration could result in higher levels particularly at healthy sites where GCF volume is very low (Curtis et al. 1988, Emingil et al. 2000). Moreover, it has been stated that a greater increase of GCF volume may lead to decreased cytokine concentration in GCF samples (Tsai et al. 1998). Therefore, high GCF levels of TGF-$\beta_1$ concentration detected at healthy sites might be related to small amount of GCF volume.

TGF-$\beta_1$ is a pivotal mediator in resolution of gingival inflammation and initiation of wound healing (Sodek & Overall 1992). It plays an important role in wound healing by stimulating fibroblast proliferation, increasing the synthesis of extracellular matrix molecules and inhibitors of MMPs as well as inhibiting matrix-metalloproteinase synthesis (Laiho & Keski-Oja 1989, Matsuda et al. 1992). Wrana et al. (1986) have demonstrated that TGF-$\beta_1$ could increase the matrix synthesis of gingival fibroblasts by 1.7-fold at 1 ng/ml concentration. This cytokine could also induce formative fibroblast phenotype, which could synthesize connective tissue matrix (Overall et al. 1991). It was hypothesed that alteration in matrix composition during inflammation and wound healing is partly regulated by an altered phenotype of matrix producing cells and partly by the altered response of these cells to TGF-$\beta_1$ (Hakkinen et al. 1996). van der Zee et al. (1997) have stated that TGF-$\beta_1$ has a central role in regulation of collagen metabolism in physiologic as well as pathologic conditions like periodontitis. Authors have proposed that TGF-$\beta$ could contribute to building of an equilibrium between destructive and reparatory factors by inhibiting collagen matrix destruction in phases of remission and healing during periodontal disease. When the microbial load sustains as in chronic periodontitis there will be a state of equilibrium. Following the elimination of microbial factors inflammation will resolve and tissue repair will be completed in time (Steinsvoll et al. 1999). In the present study, both total amount and concentration of GCF TGF-$\beta_1$ increased following both adjunctive SDD and placebo therapy at 3 months. The increase in cytokine levels following elimination of

microbial factors is probably directed towards the rapid and effective restoration of the periodontium (Ejeil et al. 2003). Although statistically not significant, at 6 months GCF, TGF-$\beta_1$ concentration in both groups found to be higher when compared with baseline levels. This might represent a constant wound healing and cellular activity in periodontal tissues.

In the present study, we have demonstrated that GCF TGF-$\beta_1$ total amount and concentration in adjunctive SDD group was significantly higher than that of placebo group at the end of medication period. TCs are known to increase collagen production in connective tissue (Schneir et al. 1990, Sasaki et al. 1992, Craig et al. 1998). It is well documented that TGF-$\beta_1$ could increase the production of various connective tissue matrix constituents including collagen (Chen et al. 1987, Fine & Goldstein 1987, Varga et al. 1987, Westergren-Thorsson et al. 1990, Matsuda et al. 1992). It could be interesting to speculate that the increase in collagen synthesis following TC application might be partly regulated by TGF-$\beta_1$. In an in vitro study Shlopov et al. (2001) have detected that doxycyline increased mRNA levels of TGF-$\beta$ receptors in osteoarhtritic cartilage cultures. Various in vitro studies have demonstrated that TGF-$\beta_1$ application reduced synthesis of several MMPs including MMP-1, -2, -8, -9 and -13 (Shlopov et al. 1999, Palosaari et al. 2000, Vaday et al. 2001, Martelli-Junior et al. 2003). Therefore, it could be proposed that SDD could contribute to connective tissue healing and collagenous matrix production via increasing TGF-$\beta_1$, which regulate collagenase activity.

During periodontal diseases TGF-$\beta_1$ could alternate between pro-inflammatory or anti-inflammatory roles related to the nature of host response. During early phases of inflammation a pro-inflammatory role is attributed to TGF-$\beta_1$ before shifting to anti-inflammatory role (Skaleric et al. 1997) However, at what stages of periodontal disease and healing TGF-$\beta_1$ exerts pro or anti-inflammatory role remains to be elucidated. In an human experimental gingivitis study, Wright et al. (2003) demonstrated that GCF TGF-$\beta_1$ total amount decreased nearly to healthy levels 14 days after application of prophylaxis following a 21 day of non-brushing period. On the other hand, GCF TGF-$\beta_1$ concentration did not show significant changes during this experimental study period. In the present study both total amount and concentration of GCF TGF-$\beta_1$ increased following both adjunctive SDD and placebo therapy at 3 months. The contrast in findings of Wright et al. and our study might result from different destructive features of gingivitis and chronic periodontitis. Therefore, considering the increase instead of decrease of cytokine levels after resolution of inflammation it could be speculated that in chronic periodontitis anti-inflammatory and reparatory properties of TGF-$\beta_1$ might be influential to its pro-inflammatory properties. Moreover, since gingivitis is not accompanied with bone loss, this could likely reflect alveolar bone remodeling mediated by TGF-$\beta_1$ (Sodek & Overall 1992). However, it remains unclear whether levels of this cytokine decreases at early stages of healing following non-surgical treatment of chronic periodontitis because of resolution of gingival inflammation.

The results of the present study indicate that adjunctive SDD therapy improves the clinical parameters and increases the GCF TGF-$\beta_1$ levels in patients with severe, generalized chronic periodontitis. It could be suggested that beneficial effects of adjunctive SDD could be maintained after cessation of the treatment. On the other hand, the efficacy of SDD at the biochemical level is likely continuing as long as the agent is used. This likely indicates that biochemical improvements could occur before clinical improvements. Therefore, cyclical regimen of SDD with 3-month intervals could be recommended in order to improve biochemical markers. To our knowledge, this is the first study investigating the effect of SRP and SRP plus SDD on GCF TGF-$\beta_1$ levels in chronic periodontitis subjects. The ability of doxycycline to increase TGF-$\beta_1$ might provide another mechanism for TCs to inhibit connective tissue breakdown. Further studies addressing the the question by which pathway doxycycline increases TGF-$\beta_1$ levels will help to elucidate this proposed mechanism of action.

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