Effects of Initial Periodontal Therapy on Heat Shock Protein 70 (HSP70) and Anti-HSP70 Levels in Gingival Crevicular Fluid of Patients with Periodontitis

（歯周炎患者の歯肉溝滲出液中のヒートショックタンパク質 70（HSP70）および抗 HSP70 に対する歯周基本治療の効果）

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Preface

This article is based on a main reference paper, “Effects of Initial Periodontal Therapy on Heat Shock Protein 70 Levels in Gingival Crevicular Fluid from Periodontitis Patients” in the Journal of Clinical Medicine, and a reference paper, “Anti-heat shock protein 70 levels in gingival crevicular fluid of Japanese patients with chronic periodontitis” in the Journal of Oral Science.

Abstract

Periodontitis is an inflammatory disease of periodontium which is caused by periodontopathic bacteria. Moreover, various cytokines such as interleukin-1β, tumor necrosis factor-α and IL-6 are expressed in the inflamed periodontium. Heat shock proteins (HSPs) protect cells from abnormal conditions including inflammation, microbial infection and diseases. HSPs are classified according to their molecular weight, and one of the major ones is 70-kDa HSP (HSP70) that express in the inflamed tissues. In this study, enzyme-linked immunosorbent (ELISA) assay was applied to measure the levels of HSP70 in gingival crevicular fluid (GCF) from two periodontal pockets in each of 10 patients with Stage III, Grade B periodontitis. Sites with probing pocket depth (PPD) of ≤3 mm named the healthy control (HC) sites, and sites with PPD of ≥5 mm

named the diseased sites. HSP70 levels in GCF were expressed higher at diseased sites than at HC sites and decreased after initial periodontal therapy at diseased sites. These results suggest that the association of HSP70 with stage of periodontitis.

One of the causes of periodontal disease is thought to be an imbalance in the expression of HSPs and anti-HSP antibodies. The objective of the second study was to measure the anti-HSP70 antibody levels in GCF from two gingival sulci in each of nine patients with chronic periodontitis (diseased site): one healthy control (HC) site with a PPD of ≤3 mm and one diseased site with a PPD of ≥5 mm. Anti-HSP70 antibody levels in GCF were higher at HC sites than at diseased sites. Moreover, the anti-HSP70 antibody levels were found to increase after initial periodontal therapy at both HC and diseased sites. These results suggest an association of anti-HSP70 antibody with periodontitis.

Introduction

Periodontitis is a common disease which is the inflammation of periodontium caused by oral microorganisms (1). Numerous cytokines have been detected in the gingival crevicular fluid (GCF). Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels have been found in increased in GCF from inflamed gingival tissues (2,3). IL-1β, matrix metalloproteinase-8 and IL-8 levels in GCF were significantly higher in diseased sites than in healthy subjects, and those levels in GCF were significantly decreased after

periodontal treatment (4). In a recent study, GCF volume and IL-1β levels in GCF reflected the disease severity and that these parameters were suggested to be better than probing pocket depth (PPD) and bleeding on probing (BOP) as markers of gingival inflammation (5). Therefore, it could be helpful to investigate the inflammatory cytokine levels in GCF for diagnosis of the active phase of periodontal disease. Heat shock proteins (HSPs) act as molecular chaperones which are enhanced proteins for protecting cells immediately after cells are exposed to heat shock stress (6). The 70-kDa HSPs (HSP70s) comprising various isoforms involve in a number of human pathologies, ranging from cancer to neurodegenerative diseases (7,8). HSP70 levels were higher in protein extract from inflamed gingiva collected during periodontal surgery, and HSP60 and HSP65 levels were higher in serum from the patients with periodontitis (9). In addition, expression levels of IL-1β, IL-8 and HSP70 were increased in GCF from patients with periodontitis compared with healthy subjects (10). Serum concentration of chitinase-3-like-1 (YKL-40) which is a novel marker of acute and chronic inflammation was significantly higher in patients with periodontitis and diabetes compared to healthy groups. However, GCF concentration of YKL-40 was similar in patients with periodontitis, diabetes and healthy subjects, whereas YKL-40 levels were significantly increased in diabetes patients with periodontitis (11). Therefore, HSP70 can be considered potentially as a marker for the severity of periodontal disease. However, there is no report on changes in HSP70 concentration in GCF before and after initial periodontal therapy, and its correlation with clinical parameters. The aim of this study was to elucidate the HSP70 levels in GCF from patients with periodontitis, and compare their concentrations in GCF at first visit, after initial periodontal therapy, and 3 months follow-up.

Wound healing is a complicated biological process involving sequential molecular and cellular events that are regulated spatially and temporally. Heat shock proteins (HSPs) are a protein family produced in response to stress conditions, and serve as a sensitive biological marker of thermal stress. HSPs are present in all organisms, being expressed in response to several environment stressors to protect cells from damage, and promoting a variety of biological processes by acting as molecular chaperones under non-stressed conditions (12). HSPs are classified according to their molecular weight, and the HSP70 family has been intensively investigated. Among the HSP70 family members, stress-inducible HSP72 and constitutively expressed HSP73/HSC70 are the best known (13,14). Anti-HSP70 antibodies are found in smokers and patients with Graves' disease (15). Patients with uveitis have circulating anti-HSP70 antibodies whose levels may depend on disease severity (16). The serum levels of various HSPs antibodies, including anti-HSP70, are reportedly higher in patients with dilated cardiomyopathy than in healthy controls (17). The relationship between the level of anti-HSP70 antibody and several types of vascular disease suggests that HSP70 might contribute to the pathogenesis and progression of atherosclerosis (18). It has been suggested that the plasma levels of anti-HSP70 or anti-HSP71 antibodies might be associated with hypertension and harsh working conditions, and are increased in patients with severe heat exhaustion (19,20). It

has been reported that patients with graft-versus-host disease after allogeneic stem cell transplantation possess circulating anti-HSP70 and anti-HSP90 antibodies (21). HSP70 is thought to be a potential autoantigen in multiple sclerosis (22), and high levels of expression of HSP27, HSP70 and HSP73 in myelin may act as an additive immune target involved in progression of the disease (23,24). Anti-HSP70 antibody could also be a causal factor of schizophrenia, especially in patients who have not been medicated for it (25,26). Blood levels of anti- HSP70 antibody and formation of HSP70-antibody complexes in the placenta may induce preterm birth (27). Moreover, the levels of HSP70 and its antibody can be used as diagnostic and prognostic indicators in patients with gynecological malignancies (28,29). Inflamed periodontal tissue shows marked down-regulation of HSP70 family members (30), and it has been reported that levels of anti-HSP70 antibody in periodontitis patients did not change significantly during periodontal treatment for 6 months (31). This relationship between HSP70 family members and periodontal inflammation suggests that HSP70 and its antibody might contribute to the pathogenesis and progression of periodontitis. The purpose of the present study was to clarify the levels of HSP70 and anti-HSP70 antibody in GCF from both diseased and healthy control (HC) gingival sulci in patients with periodontitis, and to consider the possible significance of HSP70 and anti-HSP70 antibody levels in relation to periodontal

disease severity.

Materials and Methods

Study population

For the first study, 10 patients with Stage III, Grade B periodontitis (mean age, 53.9 ± 3 years) at Nihon University Hospital School of Dentistry at Matsudo, Japan were recruited and then received initial periodontal therapy, including oral hygiene instruction, scaling and root planing, and professional mechanical tooth cleaning. The stage of periodontitis was assessed clinically based on clinical attachment loss (CAL), radiographic bone loss (BL), periodontal history of tooth loss (PTL), probing pocket depth (PPD), BOP, furcation involvement (Class II or III), plaque index (PlI), and gingival index (GI) (32-34). A CP11 probe (Hu-Friedy, Chicago, IL, USA) was used to measure the PPD and CAL. They were diagnosed when BL affected the middle third of the root or beyond and CAL was 5 mm or more. If PTL was four teeth or less, then the diagnosis was stage III. When the patient's previous periodontal records were available, the rate of periodontitis progression in the previous five years could be estimated. If progression was <2 mm, then the diagnosis was Grade B periodontitis (35). GCF samples were taken from two periodontal PPD sites (shallow PPD of ≤3 mm were named the healthy control (HC) sites and deep PPD of ≥ 5 mm were named the diseased sites) for each patient. This study was approved by the ethics committee at Nihon University School of Dentistry at Matsudo (EC17-017, EC18-17-017-1). Written informed consent was obtained from all patients after all of the details of the investigative approach had been explained to them. Ten

patients were confirmed physically healthy and had no experience of periodontal treatment or antibiotic treatment for at least three months prior to participation in this study. None of the patients were smokers.

For the second study, 9 patients with chronic periodontitis (mean age, 62.8 ± 9.1 years) were recruited. All received initial periodontal therapy at Nihon University Hospital School of Dentistry at Matsudo, Japan. The ethics committee at Nihon University School of Dentistry at Matsudo approved the study (EC17-017, EC18-17-017-1). Periodontitis was assessed clinically in terms of PPD, CAL, BOP, PlI and GI (32,33). Patients were defined as having periodontitis if they had at least two sites with a PPD of ≥5 mm and an attachment loss of ≥5 mm (36). All patients were physically well and had no history of periodontal therapy or antibiotic treatment for at least 3 months prior to participation in the study.

Sample size

Sample size was determined by the statistical software Easy R (EZR) on R commander Ver.1.27 (Tokyo, Japan) (37). It was calculated that six samples (samples were taken from two sites per patient) were necessary for each group (HC and diseased sites) to reach 80% power at 5% level of significance. Therefore, the number of samples taken was appropriate.

For the second study, to confirm the required sample size for this study, 80% power with a two-sided comparison ($\alpha = 0.05$), the predicted difference in the mean value between two groups (550 ng/ml) and the standard deviation of two groups (400 ng/ml) were set and calculated. The required sample size for each group was 9. Therefore, the number of samples taken was appropriate.

Clinical protocol

1st study

Clinical examinations were performed three times (first visit (1st), second examination (2nd) after initial periodontal therapy, and third examination (3rd) at a three month follow-up after initial periodontal therapy) by two periodontal specialists (H.T. and Y.O.). Average period of initial periodontal therapy was three months. At the point of each examination (1st, 2nd, and 3rd), GCF was collected using Periopaper subsequent to removal of supragingival plaque with a sterile curette. The GCF volume (μl) was measured using a Periotron 4000 (Oraflow, New York, NY, USA). GCF samples for the 1st, 2nd and 3rd visits were taken before the periodontal therapy on the same day. Periopaper was then inserted into the pocket for 30 s each time. GCF samples were kept in Eppendorf tubes from two periodontal sites (≥ 5 mm (deep PPD site; Diseased) and ≤3 mm (shallow healthy control site; HC)) from each patient and stored at −80 °C until measurement of HSP70 levels (38).

2nd study

Clinical examinations were performed 4 times (first visit [1st], 2nd examination [2nd] after supragingival scaling, 3rd examination [3rd] at 3 months after scaling and root planing (SRP), and 4th examination [4th] at 6 months after SRP under infiltration anesthesia). GCF was collected 4 times (1st, 2nd, 3rd, and 4th). GCF samples were pooled in Eppendorf tubes from two periodontal sites (≥5 mm [deep PPD site; Diseased] and ≤3

mm [shallow healthy control site; HC]) in each of the 9 patients and stored at −80ºC until measurement of anti-Hsp70 antibody levels (38).

Enzyme-Linked Immunosorbent Assay (ELISA)

1st study

Concentrations of HSP70 in GCF samples were measured by ELISA using the Human HSPA4 (HSP70) ELISA kit (Invitrogen) according to the manufacturer's instructions. GCF samples were dissolved in 300 µl of Sample Diluent C in the ELISA kit. Diluted samples (100 µl) were incubated for 2.5 h in an anti-human HSP70 pre-coated 96-well strip plate. After a wash, biotinylated antibody was added to the wells for 1 h. After a wash, streptavidin-HRP solution was added to the wells for 45 min. After washing, TMB substrate was added to the wells for 30 min. Color development was stopped and the optical density at 450 nm of each well was measured within 30 min using a microplate reader. All measurements were performed in duplicate and the concentrations of HSP70 were expressed in ng/ml.

2nd study

Concentrations of anti-Hsp70 antibodies in GCF samples were measured by ELISA using the Anti-Hsp70 IgG/A/M (human) ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) in accordance with the manufacturer's instructions. Briefly, GCF samples collected in the Periopaper strips were dissolved in 750 µl of Sample Diluent 2 provided in the kit. The diluted GCF samples (100 µl) were then incubated for 2 h in recombinant human Hsp70 protein pre-coated microplate wells. After a wash to eliminate unbound

substances, hydrogen peroxidase-conjugated anti-human IgG, IgA and IgM goat sera were added to the wells. After washing away any unbound conjugate, tetramethylbenzidine substrate solution was added to the wells for 15 min. Color development was stopped within 30 min, and the optical density of each well was determined using a microplate reader at 450 nm. All measurements were performed in duplicate and the concentrations of anti-Hsp70 antibodies were expressed as ng/ml.

Statistical analysis

Clinical parameters are shown as mean ± standard error (SE). The significant differences between each examination for clinical parameters, GCF volume, and HSP70 levels among the groups were determined by two-way ANOVA. For the second study, significance of differences between the examinations for clinical parameters, GCF volume and anti-Hsp70 antibody levels among the groups were determined by Steel-Dwass test, and presented as the median and interquartile range. Difference in BOP at the 1st to 3rd examinations were analyzed by a Chi-squared test. The level of significance was adjusted at 5%. Four steps Ekuseru-Toukei (the publisher OMS Ltd.) was used for statistical analyses.

Results

1st study

The patient characteristics such as age, sex, PPD, CAL, PlI, GI, and BOP distributions for the 10 patients in this study are listed in Table 1. Average PPD and CAL at the HC sites (PPD ≤ 3 mm) were 2.7 ± 0.2 mm and 3.9 ± 0.4 mm, and at the diseased sites (PPD

≥ 5 mm) were 6.5 ± 0.5 mm and 7.7 ± 0.7 mm, respectively. GI and BOP scores at the diseased sites (1.7 ± 0.2 and 80%) were higher than those at the HC sites (0.3 ± 0.2 and 0%). PlI at the HC and diseased sites were the same score (1.1 ± 0.2). The concentrations of HSP70 in GCF from the HC and diseased sites at each point of examination during initial periodontal therapy are shown in Table 2. The average HSP70 level at the 1st visit in GCF from diseased sites was significantly higher than HC sites. Moreover, the concentration of HSP70 at diseased sites was significantly decreased at 3rd examination (a three month follow-up after initial periodontal therapy) as compared to the 1st examination. The concentration of HSP70 at HC sites did not change through the periodontal therapy (1st, 2nd, and 3rd examinations) (Table 2). Changes in five kinds of clinical parameters (PPD, CAL, PlI, GI, and BOP scores) at the HC and diseased sites during initial periodontal therapy are listed in Tables 3 and 4. At HC sites, PlIs were significantly decreased at the 2nd and 3rd examinations as compared to the 1st examination (Table 3). On the other hand, PPD and PlI at diseased sites were significantly decreased at the 2nd and 3rd examinations compared to the 1st examination (Table 4). Furthermore, GI and BOP scores at diseased sites were significantly decreased at the 3rd examination compared to the 1st examination. GCF volumes from HC and diseased sites were measured by Periotron 4000 during the course of periodontal therapy (Table 5). GCF volumes from HC and diseased sites did not change during the periodontal therapy, however, the volumes of GCF from the diseased sites were significantly higher than those in the HC sites at the 1st and 2nd visits (Table 5).

2nd study

The age, sex, PPD, CAL, PlI, GI, and BOP distributions for the 9 patients are listed in Table 6. Average PPD and CAL at the HC sites (PPD ≤3 mm) were 2.9 ± 0.1 mm and 3.2 ± 0.2 mm, respectively. In contrast, average PPD and CAL at the diseased sites (PPD ≥5 mm) were 6.1 ± 0.3 mm and 7.9 ± 0.9 mm, respectively. PlI, GI, and BOP scores at the diseased sites (2.2 ± 0.4, 1.8 ± 0.1 and 78%, respectively) were higher than those at the HC sites (0.3 ± 0.2, 0 and 0%). Changes in the concentrations of anti-HSP70 antibody in GCF from the HC and diseased sites during initial periodontal therapy are shown in Table 7. The median anti-HSP70 antibody level at the 4th visit in GCF from HC sites was significantly higher than that from diseased sites. However, the concentrations of anti-HSP70 antibody at HC and diseased sites did not change significantly during the periodontal therapy (1st, 2nd, 3rd, and 4th examinations) (Table 7). Changes in the PPD, CAL, PlI, GI, and BOP scores at the HC and diseased sites during initial periodontal therapy are listed in Tables 8 and 9. At HC sites, clinical parameters remained almost unchanged during the periodontal therapy (Table 8). In contrast, at diseased sites, PPD was reduced from 6 (5-8) mm at the 1st examination to 4 (3-6) mm at the 4th ($P < 0.05$). Furthermore, BOP scores were reduced from 78% at the 1st examination to 56% at the 2nd, and 33% at the 3rd and 4th ($P < 0.05$) (Table 9). Changes in the volume of GCF from HC and diseased sites during the course of periodontal therapy are shown in Fig. 1. Although GCF volume at these sites did not change significantly, there were significant differences in between HC and diseased sites at the 1st and 3rd visits (Fig.1).

Discussion

In the first study, we have shown that there was a significant difference in HSP70 concentration in GCF between HC and diseased sites at the first visit. The concentration of HSP70 in GCF from diseased sites was significantly decreased at the three month follow-up after initial periodontal therapy (3rd examination; Table 2). At HC sites, PlI was significantly decreased at the 2nd and 3rd examinations (Table 3). At diseased sites, PPD and PlI were significantly decreased at the 2nd and 3rd examinations, whereas GI and BOP were significantly decreased only at the 3rd examination as compared to the 1st visit (Table 4). These results suggest that initial periodontal therapy is effective in improving inflammation of periodontal tissues and there is an association between the level of HSP70 and periodontitis. In addition, improvements of GI, BOP, and HSP70 levels were found to take longer than improvements of PPD and PlI. Intracellular HSP levels are elevated immediately after exposed to stresses such as high temperature. HSPs are involved in the maintenance of cellular homeostasis and protein repair in damaged cells (39). However, there are several unclear points in the relationship between HSPs and periodontitis. Inflammatory periodontal pockets have a higher temperature than healthy pockets (40). Inflammatory cytokines, such as IL-1, TNF-α, and INF-γ, are produced in inflamed periodontal tissues (41), and they might act as stressors to induce the expression of HSPs. Lipopolysaccharide and IL-1 increased hyperthermia-induced HSP70 in monocyte/macrophage-like RAW264.7 cells (42). However, one study described how HSP70 dramatically down-regulated in the inflamed periodontal tissues (43). Another study showed that GCF volume at the first visit decreased significantly after initial periodontal therapy (44). However, in this study, GCF volumes from HC and diseased sites did not change during periodontal therapy (Table 5), although the GCF volumes from diseased sites at the 1st and 2nd visits were significantly higher than the

GCF volumes from HC sites (Table 5). Therefore, further study is necessary to elucidate the involvement of HSP70 in the onset and progression of periodontitis. Stress and smoking are environmental factors for periodontitis (45,46). Several studies have shown that smoking has an adverse effect on the incidence and progression of periodontitis (46). In the synovial tissues of smokers with rheumatoid arthritis (RA), HSP70 levels were significantly higher than in the synovial tissues of non-smokers with RA (47). Therefore, smoking could increase the expression of HSP70. There are several studies describing the association between HSP70 and cancer. Malignant cells, such as osteosarcoma derived cells, expressed higher levels of HSP70 during tumor progression compared to normal cells (48). Moreover, HSP70 has been assessed as a marker for oral epithelial dysplasia such as oral leukoplakia (49). Therefore, various studies have been conducted to develop the HSP70 inhibitors for cancer therapy (50). In the first study, GCF volumes from the diseased sites were significantly higher than those in the HC sites at the 1st and 2nd visits. HSP70 concentration in GCF from diseased sites was significantly higher than the concentration of HSP70 from HC sites at the 1st visit. Moreover, the HSP70 concentration at the 1st visit was significantly decreased at the three month follow-up after initial periodontal therapy together with clinical parameters, such as PPD, GI, PlI, and BOP. These results suggest that the HSP70 concentration could become an appropriate indicator for the healing process of periodontitis.

In the second study, it has been shown for the first time that the levels of anti-HSP70 antibody in GCF from HC sites were significantly higher than those at diseased sites at the 4th visit during periodontal therapy in patients with periodontal disease. The concentrations of HSPs are up-regulated rapidly when cells are exposed to environmental stressors such as elevated temperature. They are involved in crucial physiological

processes, and in protein repair in damaged cells (39). It is well known that several types of proinflammatory cytokine are produced in inflamed periodontal tissues (40, 41), and might act as stress factors to increase the level of endogenous HSPs. However, HSC70, HSP70-2 and heat shock protein family A (HSP70) member 4 are markedly down-regulated in inflamed periodontal tissues. These intracellular HSP70 family proteins facilitate the folding of newly synthesized proteins and prevent protein aggregation (43).

In the second study, it has examined the protein levels of extracellular anti-HSP70 antibody in GCF from two gingival sulci (HC and diseased sites] in patients with periodontal disease. The levels of anti-HSP70 antibody in GCF were significantly higher at HC sites than at diseased sites at the 4th visit. Moreover, anti-HSP70 antibody levels were increased after initial periodontal therapy in both HC and diseased sites. Although these results suggest an association of anti-HSP70 antibody with periodontitis, the findings are limited and further studies will be needed. Toll-like receptor (TLR) agonists, endotoxin, and heat-killed streptococcus activate the expression of HSP70 *in vitro* (51) and *in vivo* (52). If endotoxemia and fever persist in patients with sepsis, expression of HSP70 might be often observed (53, 54). The expression patterns of HSP70 and HSP25 after CO_2 laser irradiation of gingival tissues suggest that HSP70 mainly confers cell protection, whereas HSP25 is involved in the promotion of repair and cell protection (55). Low-intensity pulsed ultrasound has been reported to increase the expression of HSP70 in gingival epithelial cells after flap surgery (56). These results suggest that HSP70 might participate in periodontal wound healing. Therefore, HSP70 may play a regulatory role in the aging process as a molecular chaperone, since it mitigates the effects of proteotoxic stress (57, 58). In *Caenorhabditis elegans*, knock-in of extra copies of HSP70, a homolog of mot-2 (mortalin)/mthsp70/Grp75, has been shown to extend life-span (59), whereas

knockdown of mitochondrial HSP70 induces progeria-like phenotypes (60). Patients with autoimmune diseases and rheumatism frequently have auto-antibodies against HSPs. Anti-HSP60 or anti-HSP70 antibodies enhance the production of IL-8 and TNF-α induced by HSP60 or HSP70 in human peripheral blood monocytes and monocytic cells (61). Serum levels of both HSP70 and its antibody are increased in Behçet's disease and both can be predictive of acute coronary syndrome (62, 63). Anti-HSP70 antibody levels are associated with nascent metabolic syndrome and are significantly higher in healthy subjects than in patients with type 1 diabetes. Therefore, the serum level of anti-HSP70 antibody might be novel marker of protection against chronic diabetic complications (64, 65). In this study, anti-HSP70 antibody levels in GCF were significantly higher at HC sites than at diseased sites. Moreover, anti-HSP70 antibody levels increased after initial periodontal therapy. Therefore, it has been suggested that the level of anti-HSP70 antibody could become an appropriate indicator of the periodontitis healing process.

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Table 1 Patient characteristics

	HC sites	Diseased sites
Age	53.9 ± 3	
Gender (male/female)	2 / 8	
PPD	2.7 ± 0.2	6.5 ± 0.5
CAL	3.9 ± 0.4	7.7 ± 0.7
PlI	1.1 ± 0.2	1.1 ± 0.2
GI	0.3 ± 0.2	1.7 ± 0.2
BOP	0 (0%)	8 (80%)

HC, Healthy control; PPD, probing pocket depth; CAL, clinical attachment loss; PlI, plaque index; GI, gingival index; BOP, bleeding on probing; mean ± SE (n =10).

Table 2 Changes in the concentrations of HSP70 in GCF collected from HC and Diseased sites during the periodontal therapy

Concentration (ng/ml)	1st	2nd	3rd
HC sites	18 ± 4.99	16.73 ± 6.37	6.64 ± 3.46
	*		
Diseased sites	64.36 ± 13.74	35.1 ± 6.67	5.69 ± 1.78
		**	

GCF, Gingival crevicular fluid; HC, Healthy control; 1st, first visit; 2nd, after initial periodontal therapy; 3rd, 3 months' follow-up after initial periodontal therapy; mean ± SE (n = 10), *$P < 0.05$, **$P < 0.01$.

Table 3 Changes in clinical parameters at HC sites

	1st	2nd	3rd
PPD	2.7 ± 0.2	2.5 ± 0.2	2.5 ± 0.2
CAL	3.9 ± 0.4	3.6 ± 0.5	3.6 ± 0.4
PlI	1.1 ± 0.2 *	0.6 ± 0.2	0.6 ± 0.2 *
GI	0.3 ± 0.2	0	0.4 ± 0.3
BOP	0 (0%)	0 (0%)	2 (20%)

HC, Healthy control; 1st, first visit; 2nd, after initial periodontal therapy; 3rd, 3 months' follow-up after initial periodontal therapy; PPD, probing pocket depth; CAL, clinical attachment loss; PlI, plaque index; GI, gingival index; BOP, bleeding on probing; mean ± SE (n = 10), *$P < 0.05$.

Table 4 Changes in clinical parameters at Diseased sites

	1st	2nd	3rd
PPD	6.5 ± 0.5	4.3 ± 0.4 *	4.3 ± 0.5 *
CAL	7.7 ± 0.7	6.2 ± 0.9	5.9 ± 0.7
PlI	1.1 ± 0.2	0.5 ± 0.2 *	0.6 ± 0.2 *
GI	1.7 ± 0.2	1.2 ± 0.3	0.9 ± 0.3 *
BOP	8 (80%)	5 (50%)	4 (40%) *

1st, first visit; 2nd, after initial periodontal therapy; 3rd, 3 months' follow-up after initial periodontal therapy; PPD, probing pocket depth; CAL, clinical attachment loss; PlI, plaque index; GI, gingival index; BOP, bleeding on probing; mean ± SE (n = 10), *$P < 0.05$.

Table 5 Changes in GCF volume at HC and diseased sites during the periodontal therapy

GCF (μL)	1st	2nd	3rd
HC sites	0.92 ± 0.2	0.47 ± 0.2	0.66 ± 0.2
	*	*	
Diseased sites	2.41 ± 0.5	1.6 ± 0.3	1.31 ± 0.3

GCF, Gingival crevicular fluid; HC, Healthy control; 1st, first visit; 2nd, after initial periodontal therapy; 3rd, 3 months' follow-up after initial periodontal therapy; mean ±SE (n = 10), *$P < 0.05$.

Table 6 Patient characteristics

	HC sites	Diseased sites
Age	62.8 ± 9.1	
Males	2 (22%)	
Females	7 (78%)	
PPD	2.9 ± 0.1	6.1 ± 0.3
CAL	3.2 ± 0.2	7.9 ± 0.9
PlI	0.3 ± 0.2	2.2 ± 0.4
GI	0	1.8 ± 0.1
BOP	0 (0%)	7 (78%)

mean ± SE (n = 9)

Table 7 Changes in the concentrations of anti-Hsp70 antibody in GCF collected from HC and Diseased sites during the therapy

Concentration (ng/ml)	1st	2nd	3rd	4th	
HC sites	1001.4 (520.2-4388)	1466 (276.8-2012.2)	1311(139.5-2913)	1595.9 (305.2-2845.2)	*
Diseased sites	718.2 (33.3 -1068.5)	719.2 (173.9-1143.9)	690.6 (404.2-2740.1)	1030.9 (356.2-1560.5)	

1st, first visit; 2nd, 2nd examination after supragingival scaling; 3rd, 3 months after SRP; 4th, 6 months after SRP;
*$P < 0.05$; median and interquartile range ($n = 9$)

Table 8 Changes in clinical parameters at HC sites

	1st	2nd	3rd	4th
PDD	3 (2-3)	3 (2-4)	3 (2-4)	3 (2-3)
CAL	3 (2-4)	3 (2-4)	3 (2-5)	3 (2-5)
PlI	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)
GI	0	0 (0-1)	0 (0-2)	0 (0-2)
BOP	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Median and interquartile range (n = 9). Changes in BOP at the 1st, 2nd, 3rd and 4th examinations were analyzed by chi-squared test.

Table 9 Changes in clinical parameters at Diseased sites

	1st	2nd	3rd	4th
PDD	6 (5-8)	5 (4-7)	5 (5-8)	4 (3-6)*
CAL	7 (6-14)	7 (4-11)	6 (4-13)	7 (4-10)
PlI	3 (0-3)	3 (0-3)	2 (0-3)	2 (0-3)
GI	2 (1-2)	2 (0-2)	1 (0-2)	1(0-2)
BOP	7 (78%)	5 (56%)	3 (33%)*	3 (33%)*

Median and interquartile range ($n = 9$) compared with 1st, *$P < 0.05$. Differences in BOP at the 1st, 2nd, 3rd and 4th examinations were analyzed by chi-squared test.

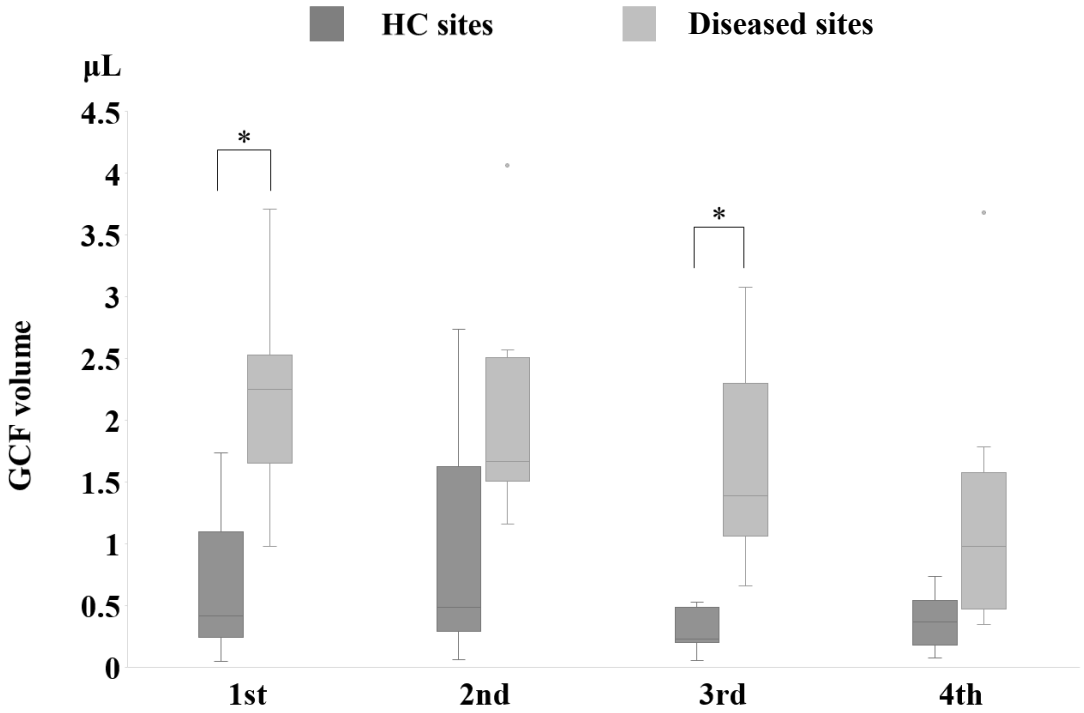

Fig. 1 Box plot showing changes and variations in GCF volume at HC and Diseased sites during the periodontal therapy (median and interquartile range; $n = 9$). $^{*}P < 0.05$