

EFFECT OF ADJUNCTIVE USE OF SIMVASTATIN GEL IN MANAGEMENT OF PERIODONTITIS ON THE CREVICULAR FLUID CALPROTECTIN LEVELS

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ABSTRACT

Background: Calprotectin (CLP) is a potential marker in inflammation; therefore, this study was designed to compare crevicular fluid levels of CLP among patients having periodontitis and healthy subjects, and also alterations in these levels after initial periodontal therapy and topical application of simvastatin (SMV) in periodontitis.

Methods: Sixty-nine subjects were divided into 2 groups; 15 were healthy controls (group I), and 54 patients were diagnosed as having periodontitis. Those patients were classified equally, as group II treated conventionally and group III treated conventionally as well as application of 1.2% SMV gel. CLP levels and periodontal parameters were detected at baseline and six weeks after periodontal therapy for the periodontitis patients.

Results: CLP levels were higher in patients having periodontitis than, in healthy subjects at baseline. Additionally, periodontal parameters and CLP levels reduced after treatment in both studied groups; however, there was more reduction in these indicators in group III than in group II. Moreover, there was a significant difference in these levels between group I and each of group II and III before and after treatment and also between groups II and III after treatment. However, there were no statistically significant differences in clinical parameters between both groups II and III after treatment, except in periodontal probing depth and clinical attachment level in group III.

Conclusion: Crevicular CLP levels were elevated in periodontitis patients, and significantly decreased following the adjunctive use of SMV. Thus, alteration in these levels could reflect the periodontal inflammation, as well as the clinical treatment outcome.

KEYWORDS: Calprotectin, gingival crevicular fluid, periodontitis

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INTRODUCTION

Periodontitis is a chronic immuno-inflammatory, microbial disease of the supporting tissues of the teeth and ultimately leads to their loss. Although, bacterial plaque accumulation is an initiating factor, the disease severity and progression as well as the clinical treatment efficacy are affected by the host immune responses which are afflicted by many environmental and behavioral cofactors^[1]. Furthering, this microbial infection strafes the host and triggers the local and systemic immuno-inflammatory responses. Thus, periodontitis results in destruction of the periodontal structures and also effectuates other tissues in the body by increasing the levels of various circulating inflammatory molecules such as, cytokines; which are key inflammatory mediators in periodontal disease; hormones, chemokines and others^[2].

In periodontal infection, large number of polymorphonuclear leukocytes (PMNs); which can effectively defend against invasive periodontopathogens; arrive the involved area, release a variable type of bioactive enzymatic molecules and proteins, and perform an antibacterial function which is protective by intent. However, once they are released in excess, they can result in considerable destruction of the adjacent cells and tissues and also aggravation of the host inflammatory responses^[3]. Actually, there are abundant anti-neutrophil cytoplasmic antibodies such as; myeloperoxidase, elastase, proteinase 3, cathepsin G, and lactoferrin as well as calprotectin^[4].

Calprotectin (CLP), one of the S100/calgranulin family and also called myeloid-related protein, is an abundant heterodimeric calcium and zinc binding cytosolic protein composed of S100A8 and S100A9^[4]. It is also mainly existing in many biofluids; such as, human plasma, urine, cerebrospinal and synovial fluids, as well as saliva and gingival crevicular fluid (GCF). Thus, it plays a vital role in several biological functions, including differentiation and proliferation of cells, inflammation, immunoregulation and oncogenesis^[5]. In addition, it is fundamentally

expressed in monocytes/macrophages and neutrophils in which its concentration in their cytoplasm is about 40-60%^[4]. Other cells can produce CLP when they are activated under certain conditions; such as, epithelial cells, microvascular endothelial cells, fibroblasts, keratinocytes, and osteoclasts^[6,7]. Moreover, CLP might modulate the immune-inflammatory responses^[8] as extracellularly, it could promote the chemotaxis and recruitment of PMNs and the release of inflammatory mediators; and also exhibit anti-inflammatory impacts in certain circumstances. Whereas intracellularly, CLP could harmonize and regulate the cytoskeleton; and also play a role in the protection against invading pathogenic microorganisms and the metabolism of arachidonic acid^[9].

Therefore, this protein had been a specific target for many scientists; especially who hypothesized that it had a role in the pathogenesis of periodontal diseases as its concentration could signify the severity of the inflammation in patients with those diseases^[10] and the initial periodontal therapy could also reduce its levels^[11]. In addition, CLP concentration in saliva and serum was associated with periodontitis^[12] and its levels in the GCF as well as plasma were more elevated in periodontitis patients than that in healthy controls^[13]. More recently, Gao et al.^[14] reported the highest levels of both plasma and crevicular fluid CLP in patients with chronic periodontitis and type 2 diabetes mellitus, followed by chronic periodontitis, whereas, the lowest levels were in healthy controls, with significant reduction after initial periodontal therapy.

Considering the periodontal therapy, mechanical removal of local deposits is considered the most suitable and consistent way for arresting the disease progression^[15]. However, this kind of therapy often results in unsatisfactory findings as being evident by residual inflammatory signs or periodontal pockets because of the invasive capability of the periodontal pathogens^[16]. Thus, several multiform of systemic and local adjunctive therapeutic approaches have been suggested and tried^[17]. Additionally, the ad-

adjunctive use of local drug delivery systems (LDDS) to scaling and root planing (SRP) is a promising favorable modality of treatment and resulting in more compliance, with less side effects and chances of developing bacterial tolerance to medical agents in comparison with those used systemically ^[18].

Among these LDDS are gel formulations that are popular in dental practice due to their high biocompatibility and bioadhesivity, in addition to improved efficacy of the drug as they could keep it in place prolonging its action without the risk of irritation or allergic reactions ^[19]. Simvastatin (SMV), a specific competitive inhibitor of 3- hydroxyl- 2- methyl- glutaryl coenzyme A (HMG-CoA) reductase, represents safe and effective therapeutic agent to reduce cholesterol levels in hyperlipidemia and arteriosclerosis ^[20, 21]. Several researches have studied the adjunct use of statins in periodontal treatment both *in vitro* and *in vivo*, as these agents could modulate the inflammation and immune response, and also play a role in the bone metabolism and bacterial clearance resulting in disease inactivity ^[22, 23, 24].

Regarding the disease activity, its determination is very important and several clinical indicators and parameters; such as, bleeding on probing (BOP), periodontal probing depth (PPD), clinical attachment level (CAL) and tooth mobility, have been used to appraise the periodontal tissue inflammation and destruction ^[25]. However, these clinical indices and measurements can determine neither the disease activity nor the treatment efficacy in an accurate way; hence, it is critical to find a surrogate indicator or marker ^[14]. In addition, several researches expect that the objective periodontal examination should be assessed by convenient and consistent laboratory clinical analyses based on the local inflammatory responses ^[13]. Moreover, Wei and coworkers ^[26] suggested that CLP was involved in periodontitis progression; thus, it could be a validated marker, and its levels could be periodically detected during the follow-up phase and could be used to monitor the treatment efficacy. Therefore, the current study was

proposed to evaluate the efficacy of SMV, applied topically in a gel formulation, on the GCF levels of calprotectin in periodontitis patients.

SUBJECTS AND METHODS

Sixty-nine participants were included in the present study and those consecutive subjects were recruited from the Department of Oral Medicine and Periodontology Clinic, Faculty of Dentistry, Mansoura University after taking complete medical and dental histories. All of the participants were non-smokers and none of them had received antibiotics or other medications or periodontal treatment within the last 3 months. Subjects with systemic or immunological diseases as well as other disorders that could influence the disease progression and/or the treatment response were excluded. Additionally, individuals received long-term anti-inflammatory medications or post-menopausal, pregnant and lactating females and those taking oral contraceptive drugs were also excluded from the study. The purpose of the study and the procedures were explained to all subjects prior to participation, and all participants signed an informed consent.

All participants were over 20 years and divided into 3 groups; 15 healthy subjects comprised the negative control group (group I), whereas, the other 54 participants were divided equally into 2 groups of 27 periodontitis patients, each. Those patients were diagnosed as having stage II grade A periodontitis considering the clinical and radiographic criteria of the new classification scheme [27]. The positive control group (group II) was treated conventionally by SRP only, whereas, the study group (group III) was treated by SRP followed by local delivery of 1.2% simvastatin gel. Gel application for patients in group III was performed and repeated once weekly for six weeks. At baseline and prior to any hygiene instructions or treatments, the gingival crevice fluid samples were collected from all participants, whereas, periodontal status was assessed in groups II and III. Furthering, those patients were

reevaluated in the terms of periodontal indices and measurements; such as, plaque index (PI), gingival index (GI), PPD and CAL. Both the PI and GI were evaluated at four aspects: distofacial, facial, mesiofacial and lingual^[28,29], while both the PPD and CAL were measured at 6 aspects of teeth of interest; mesiofacial, midfacial, distifacial, mesiolingual, midlingual and distolingual^[30,31].

For CLP assessment, the supragingival plaque was carefully removed with a sterile curette and the tooth surfaces were cleaned from any blood or deposits. Then, a sterile sponge and cotton rolls were used to isolate the sampling site, thus preventing its contamination by dental organisms and saliva, and allowing the site to gently dry. Using sterile paper strips, two GCF samples were collected from the deepest periodontal pocket or gingival crevice (PD < 3 mm) as one at the baseline, while, the second one 6 weeks' post-treatment by of SRP alone and with LDDS. To avoid mechanical trauma, these strips were carefully inserted until gentle resistance was felt and left there for 30seconds, with the discarding strips contaminated with blood. Strips were then placed into sterile Eppendorf tubes containing 100 ul phosphate buffer pH 7.4 and stored immediately at -80°C till time of analysis. The sample then was extracted by centrifuging at 3000 rpm for 15 min. at 4°C, and used for the assay of CLP concentrations that were tested by a commercially available ELISA kit (Bioassay Technology Laboratory, Cat. No E4010Hu R&D) according to the manufacture instruction. The calprotectin concentration in GCF was calculated from standard curve and expressed as ng/30 seconds^[32].

Statistical Analysis

The statistical analysis of studied data was done by using IBM SPSS (Statistical package for Social Science) program, Version 20.0. For parametric data, one-way ANOVA test was used for comparison of three groups' means while paired sample T test was used to compare groups before and after interventions. Chi-Square test was chosen to find the

relationship between two or more qualitative variables. Pearson correlation also was used.

RESULTS

Participants in this study were classified into three groups: Group I, n = 15; Group II, n = 27; and Group III, n = 27 with no statistically significant difference between them in either the age or gender as shown in **table 1**.

TABLE (1) Comparison of age and gender between the different groups.

Group	Gender		P value	Age (years) (Mean ±SD)	P value
	Male (N, %)	Female (N, %)			
I (n= 15)	4 (26.7%)	11 (73.3%)		36.9±7.4	
II (n= 27)	3 (11.1%)	24 (88.9%)	0.314 ^c	41.6± 5.8	0.087 ^F
III (n=27)	7 (25.9%)	20 (74.1%)		42.5 ±9.8	

SD, standard deviation, **P**; *p* value, **F**; ANOVA test, **C**; Chi square test.

Gingival crevicular levels of CLP were compared among the three studied groups before and after intervention as shown in **table 2**. The mean amounts of CLP in GCF samples of group II and group III were 256.22±31.97 and 266.70±43.97 ng/ml, respectively, and about more than 3-fold higher than the healthy individuals of the group I (37.67±16.27 ng/ml).

On the other hand, the mean calprotectin concentrations in GCF samples decreased significantly after therapy in both of group II (181.44±26.43) and group III (130.56±15.72), however, these levels were still significantly higher than those of group I of healthy controls. Moreover, there was a statistically significant difference in CLP levels between group I and each of group II and III before and after treatment (P<0.001) and also between groups II and

III after treatment ($P < 0.001$) with no statistically significant difference between groups II and III before treatment ($P = 0.546$).

TABLE (2) Calprotectin levels before and after intervention in patient and control groups.

Group	Before		After	
	Mean \pm SD	P value	Mean \pm SD	P value
I (n= 15)	73.67 \pm 16.27	$P_1 = < 0.001$	73.67 \pm 16.27	$P_1 = < 0.001$
II (n= 27)	256.22 \pm 31.97		181.44 \pm 26.43	
III (n=27)	266.70 \pm 43.77	$P_3 = 0.546$	130.56 \pm 15.72	$P_3 = < 0.001$

SD, standard deviation, **P**; p value, ANOVA test is used

P1: comparison of control group (I) with group II, **P2**: comparison of control group (I) with group III, **P3**: comparison of group II with group III

Additionally, intragroup comparison of crevicular levels of CLP demonstrated highly statistically significant differences before and after therapy in periodontitis patients of groups II and III as shown in **table 3**.

TABLE (4) Clinical assessment of patient groups before and after intervention.

	Before			After			Paired sample
	Group II	Group III	P1 value	Group II	Group III	P2 value	P value
Plaque index (Mean \pm SD)	2.50 \pm 0.17	2.42 \pm 0.24	0.217	0.82 \pm 0.11	0.79 \pm 0.13	0.221	$P_3 < 0.001$ $P_4 < 0.001$
Gingival index (Mean \pm SD)	1.76 \pm 0.17	1.79 \pm 0.14	0.519	0.41 \pm 0.09	0.38 \pm 0.06	0.119	$P_3 < 0.001$ $P_4 < 0.001$
Periodontal probing depth (Mean \pm SD)	3.54 \pm 0.23	3.46 \pm 0.24	0.212	2.51 \pm 0.19	1.70 \pm 0.19	< 0.001	$P_3 < 0.001$ $P_4 < 0.001$
Clinical attachment loss (Mean \pm SD)	2.67 \pm 0.23	2.69 \pm 0.27	0.696	2.27 \pm 0.19	1.47 \pm 0.21	< 0.001	$P_3 < 0.001$ $P_4 < 0.001$

SD, standard deviation; **p** value, **T**; t- test and paired sample t test are used. **P1**: comparison of group (II) with group III before intervention, **P2**: comparison of group II with group III after intervention, **P3**: paired samples T test before and after intervention in group II, **P4**: paired samples T test before and after intervention in group III

TABLE (3): Intra group comparison of Calprotectin levels before and after intervention.

Group	II (n= 27)		III (n=27)	
	Mean \pm SD	P value	Mean \pm SD	P value
Before	256.22 \pm 31.97	< 0.001	266.70 \pm 43.77	< 0.001
After	181.44 \pm 26.43		130.56 \pm 15.72	

Paired samples T test is used

Furthering, the characteristics of periodontal conditions in all periodontitis patients of groups II and III before and after treatment were shown in **table 4**. There were no statistically significant differences in all the periodontal parameters between the periodontitis groups before the treatment ($P_1 < 1$) and all these values were decreased after treatment in both groups. However, there were no statistically significant differences in these parameters between both groups of periodontitis patients after treatment ($P_2 < 1$), except in the PPD and CAL ($P_2 < 0.001$). Furthering, intragroup comparison of these parameters showed highly statistically significant differences before and after treatment in both groups (in group II: $P_3 < 0.001$ & in group III: $P_4 < 0.001$).

In addition, there was no statistically significant correlation between calprotectin concentrations and clinical parameters before and after treatment in the two studied groups of periodontitis patients after different interventions as shown in **table 5**.

TABLE(5): Correlation of calprotectin concentrations with clinical parameters before and after intervention in Group II and Group III.

Clinical Parameters	Group II		Group III		
	Before	After	Before	After	
Plaque index	R	-0.212	0.204	0.081	0.112
	P	0.288	0.308	0.688	0.577
Gingival index	R	0.336	-0.347	-0.047	0.202
	P	0.087	0.076	0.816	0.313
Periodontal probing depth	R	-0.303	-0.077	-0.244	0.054
	P	0.124	0.702	0.220	0.791
Clinical attachment loss	R	-0.366	0.119	-0.263	-0.078
	P	0.061	0.555	0.185	0.697

Pearson correlation is used

DISCUSSION

The present study was designed to assess if the gingival crevicular levels of CLP could be able to distinguish the healthy from periodontally affected individuals and also could be used as a valid biomarker during the course of periodontitis therapy. At the baseline, GCF calprotectin was elevated in all the studied patient groups as opposed to the control group, with non-significant difference between the patient groups in these concentrations, indicating that those patients were homogenous regarding the degree of inflammation. These results also indicating that CLP is contributed in the pathogenesis of periodontal diseases because this acute phase protein is generated from the activated polymorphonucleates within the inflamed periodontal pockets [33] and these cells entrap the GCF in specific narrow

space and liberate great volume and excessive level of CLP [34, 10]. Additionally, the lipopolysaccharide (LPS) of *Porphyromonas gingivalis* (*P.g*) increased the CLP production from neutrophils [35] and also could induce the monocytes to produce this cytosolic protein in the gingival tissue of periodontitis patients [36]. In the meanwhile, the LPS from *Aggregatibacter actinomycetemcomitans* (*A.a*), *Prevotella intermedia*, *Fusobacterium nucleatum* and *Escherichia coli* could also enhance the production of CLP from neutrophils [37].

Regarding salivary CLP, its levels could emulate and reflect the intensity of gingival inflammation as there was a gradual increase in those levels throughout the course of experimentally induced dental plaque biofilm associated gingivitis after stopping the oral hygienic regimen for 21 days [38]. However, there were contradicting results as other authors [12] reported significant higher salivary levels in patients suffering from periodontal diseases, while others detected non-significant difference in the mean salivary levels between patients with generalized aggressive periodontitis or gingivitis and healthy controls [39]. Thus, it was preferred to measure the level alterations of crevicular CLP in the present study.

In accordance with the current results, several studies [25, 34, 40, 41] reported altered crevicular levels of CLP in patients with periodontal diseases. Additionally, these levels were significantly higher in patients with aggressive and chronic periodontitis than those having gingivitis or healthy periodontium [42, 43]. Thus, it was suggested that, CLP might be an effective and relatively stable inflammatory biomarker of periodontal diseases [11, 40, 44, 13, 26]. Moreover, Kajiura and colleagues [13] reported that CLP levels in the crevicular fluid were significantly elevated in the inflammatory sites more than in the healthy sites of periodontal pockets in patients with periodontitis.

This study also evaluated the levels of crevicular fluid CLP after initial periodontal therapy with and without use of local delivery of 1.2% SMV

gel in periodontitis patients. It was found that periodontal treatment significantly decreased levels of this protein in both studied groups compared to baseline levels. However, the reduction of CLP level didn't reach the control level, perhaps owing to insufficient follow up period. Furthering, the noted reduction in CLP levels was more prominent in group III treated by LDDS compared to SRP alone in group II, suggesting a superior effect of SMV over SRP alone. Regarding the periodontal parameters, there was remarkably improvement in all parameters after treatment modalities with more significant reduction in PPD and CAL in patients treated by SMV gel. This improvement was owing to the ability of statins to reduce the production of interleukin 6 (IL-6) and IL-8 production within the epithelial cells and osteoblasts [45,46], whereas, in T-cells [47,48], they upregulate other interleukins; such as, IL-4, IL-5, IL-10 and IL-13.

These results were supported by other investigators who observed that nonsurgical periodontal therapy remarkably decreased the crevicular CLP level in patients affected by generalized aggressive periodontitis [44]. Moreover, it was reported that CLP level is a prognostic marker of the active inflammation at site and subject levels for evaluating the periodontal treatment in patients suffering from generalized aggressive periodontitis [49], chronic periodontitis [11] and also in periodontitis patients with type 2 diabetes mellitus [14]. However, there were no differences between the healthy and clinically diseased sites in patients with chronic periodontitis. On the other hand, Kajiura et al. [13] found that CLP levels were elevated significantly in the diseased inflammatory sites than in the healthy sites; thus, in the present study, the levels of CLP were evaluated by collection of GCF from the selected deepest periodontal pocket. Additionally, it was reported that the total amounts of crevicular CLP in patients with periodontitis were significantly higher than those in healthy subjects, however, levels of CLP were slightly lower in the diseased patients [50]. The difference between amounts and

concentrations of CLP was owing to the increase in the volume of GCF in inflammatory sites of periodontitis; therefore, the concentrations of crevicular CLP were evaluated in this study.

The improvement in both clinical parameters and biochemical levels in patients treated by using 1.2% SMV as LDDS resulted from the speculation that, statins played a remarkable function in the modulation of the inflammatory and immune responses by impeding the stimulation of many toll-like receptors (TLRs) 1, 2, 3, 4, 6, 7, and 9 by *A.a* lipopolysaccharide *in vivo*, thus, decreasing the possibility to evade the innate immune response [51]. It was also confirmed that statins could reduce cyclooxygenase-2, prostaglandin-E2, interferon-gamma, and C-reactive protein, in experimentally induced periodontitis [52, 51, 53]. Moreover, these drugs displayed antimicrobial activity towards some anaerobic periodontopathogens such as *A.a* and *P.g* by either killing the bacteria directly or decreasing the available cholesterol needed for bacterial protection and proliferation [54]. Additionally, most statins could arrest the periodontal tissue and alveolar bone destruction by suppressing the upregulation of matrix metalloproteinase-1 (MMP-1), MMP-8, and MMP-9 by LPS [55]. Statins could also diminish the periodontal tissue destruction caused by inflammation and gingival inducible nitric oxide synthase expression and attenuate the creation of free oxygen radicals via, a multisubunit enzyme complex, nicotinamide adenine dinucleotide phosphate oxidase [56]. Furthering, they can increase several enzymes and proteins such as; alkaline phosphatase, osteocalcin, bone sialoprotein, bone morphogenic protein-2 [57], osteopontin, and vascular endothelial growth factor, thus, inducing the osteoblast differentiation, increasing the osteogenesis, and decreasing of osteoblast apoptosis [58]. However, in this study, the short time of the follow up disabling detection of new bone formation.

Although the clinical improvement; in either patients of group II or III; there was non-significant correlation between the CLP concentrations and periodontal indices before and after treatment.

By contrast, other studies showed a significant positive correlation between the crevicular CLP levels and the periodontal parameters, such as BOP, PPD^[39,43,14], GI^[13] and also CAL^[43,14]. This discrepancy could be attributed to the short period of follow up or insufficient number of studied patients.

CONCLUSION

The current results imply that local SMV gel delivery, used as an adjunctive agent to nonsurgical initial periodontal treatment, could improve the periodontal parameters as well as significantly reduce the level of crevicular CLP in patients with stage II periodontitis compared to SRP alone, however, these levels don't return to those levels of healthy controls.

RECOMMENDATION

Further trials with longitudinal observations and adequately high numbers of patients have to be conducted for elucidation the potential role of CLP in the GCF as a predictor for the development of periodontitis. Additional studies are also needed to determine the required dose and duration of SMV for normalizing the demonstrated clinical and biochemical parameters and establishing the efficacy of simvastatin in the management of distinct forms and stages of periodontitis.

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