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Effect of adjunctive use of systemic antioxidant therapy (*N*-acetylcysteine) on soluble receptor activator nuclear factor κ B ligand levels in gingival crevicular fluid following surgical periodontal treatment for chronic periodontitis

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(Received October 5, 2016; Accepted December 15, 2016)

Abstract: *N*-acetylcysteine (NAC) is an anti-oxidant drug that has been used as a mucolytic agent and a paracetamol antidote for many years. This study was designed to determine the efficacy of the adjunctive use of NAC for periodontal treatment. Thirty subjects with moderate-to-severe chronic periodontitis were randomized to surgery with NAC (600 mg; S-NAC), surgery only (S-nonNAC), and healthy control groups. Gingival crevicular fluid (GCF) samples were obtained from all patients and sRANKL levels were determined by enzyme-linked immunosorbent assay at baseline, and 1, 3, and 7 months post-surgery. Plaque and gingival indices, probing depths, and clinical attachment levels were recorded at the same time. There was a significant reduction in probing depth at 3 months in the S-NAC group when compared to the S-nonNAC group ($P < 0.05$). However, no statistically significant differences in plaque and gingival

indices, probing depths, clinical attachment levels, and sRANKL levels in GCF were noted between the surgical treatment groups at the end of 7 months. Hence, the use of adjunctive NAC resulted in a significant reduction in probing depths in the S-NAC group when compared to the S-nonNAC group at 3 months, but no statistically significant differences in GCF sRANKL levels were observed in the sites that underwent surgical treatment with or without NAC at different time intervals.

Keywords: antioxidant; *N*-acetylcysteine; periodontal diseases; sRANK ligand; gingival crevicular fluid; enzyme-linked immunosorbent assay.

Introduction

Chronic periodontitis is considered a multifactorial infectious disease resulting from a challenge between periodontal pathogens and the host response; it presents with a slow irreversible damage of the periodontal supporting tissues over a period of time (1).

Enhanced osteoclastic activity without corresponding increase in bone formation is a major feature of inflammatory-mediated bone loss in periodontitis (2,3). At the molecular level, bone resorption is regulated by

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J-STAGE Advance Publication: October 31, 2017
Color figures can be viewed in the online issue at J-STAGE.
doi.org/10.2334/josnusd.16-0701
DN/JST.JSTAGE/josnusd/16-0701

the interplay of receptor activator of nuclear factor kappa B ligand (RANKL), receptor activator of nuclear factor kappa B (RANK), and osteoprotegerin (OPG) molecules belonging to the tumor necrosis factor ligand and receptor families (4).

RANKL stimulates osteoclastogenesis and osteoclast activity by binding to the cell-surface receptor RANK, located on osteoclasts (precursor and mature). The binding of RANKL to the extracellular RANK leads to bone resorption (5). Increased induction and expression of RANKL is related to the high concentration of proinflammatory cytokines, periodontal pathogens, and reactive oxygen species (ROS) (6). ROS cause periodontal tissue damage by various mechanisms such as lipid peroxidation, DNA damage, protein damage, and stimulation of proinflammatory cytokines released by monocytes and macrophages (7).

Various antioxidants have been used both locally and systemically to counteract ROS in periodontal diseases with beneficial results. *N*-acetylcysteine (NAC) is a derivative of the thiol-containing amino acid, cysteine, which has been demonstrated to exert anti-oxidant actions by providing sulfhydryl groups, thus acting as both a precursor of reduced glutathione and a direct ROS scavenger, and regulating the redox status in the cells (8). NAC is a widely studied water-soluble antioxidant and clinically used as a mucolytic agent as well as an antidote for paracetamol overdose (9).

The potential of NAC to suppress periodontal inflammation and alveolar bone loss in periodontal disease was investigated in an experimental periodontitis model in rats by Toker et al. (10). It was found that systemically administered NAC prevented alveolar bone loss at the ligature-induced periodontitis sites and increased osteoblastic activity in a dose-dependent manner. Furthermore, in an *in vitro* study, Kim et al. (11) examined the inflammatory responses of human gingival fibroblasts with lipopolysaccharide (LPS) obtained from *E. coli*, *Actinobacillus actinomycetemcomitans*, and *Porphyromonas gingivalis* in the presence and absence of NAC. Their findings suggest that NAC suppressed all LPS-induced inflammatory responses and exerted anti-inflammatory effects in LPS-stimulated gingival fibroblasts.

Medical literature is replete with many studies that have evaluated the role of antioxidants and their benefits in improving the outcome in systemic diseases. However, very few interventional studies have evaluated the clinical therapeutic benefits of NAC in periodontitis.

Hence, the present study was undertaken to evaluate the efficacy of NAC, an anti-oxidant drug used as an adjunct to periodontal surgery, versus surgery alone based

on an assessment of the soluble RANKL (sRANKL) levels in gingival crevicular fluid (GCF) and the clinical parameters in patients having moderate-to-severe chronic periodontitis.

Materials and Methods

Study population

A randomized, controlled, parallel group clinical study comprising 30 subjects (16 females, 14 males; age range, 35-58 years) was conducted. The patients were randomly selected from the outpatient clinic of the Oral and Dental Medicine Department, Cairo University. Twenty patients with moderate-to-severe chronic periodontitis and 10 periodontally healthy control subjects were included. The study protocol was approved by the Research Committee of the Faculty of Oral and Dental Medicine, Cairo University (2010), and consent was obtained from the participants.

The inclusion criteria for the subjects included good systemic health (assessed by a questionnaire), untreated moderate-to-severe chronic periodontitis, 35 to 60 years of age, and the presence of 15 scorable teeth (not including third molars and teeth with orthodontic appliances, bridges, crowns, or implants). Moderate-to-severe chronic periodontitis was defined as per the case definition by CDC/AAP (12). Moderate periodontitis was defined as the presence of ≥ 2 interproximal sites with ≥ 4 mm clinical attachment loss occurring at two or more different teeth or ≥ 2 interproximal sites with ≥ 5 mm probing depth on more than one tooth. Severe periodontitis was characterized as the presence of ≥ 2 interproximal sites with ≥ 6 mm clinical attachment loss, in more than one tooth, and the presence of ≥ 1 interproximal site with a ≥ 5 mm probing depth. Exclusion criteria for subjects included systemic illnesses, smoking, pregnancy or lactation, systemic drugs taken within the previous 6 months, and periodontal therapy within the previous year.

Prior to the commencement of the procedure, initial therapy was performed on all patients, which included a full-mouth SRP by universal hand curettes (SC2R/2L and SC4R/4L Columbia University Curettes, Hu-Friedy Co., Chicago, IL, USA) and with ultrasonic (piezoelectric scaler, Varios iPiezo engine, NSK, Kanuma, Japan) instrumentation as necessary, supplemented with oral hygiene instructions. All initial treatment procedures were performed by the same periodontist. One month after the subgingival debridement, the sites that did not exhibit clinical improvement following initial subgingival debridement underwent surgical periodontal treatment (modified Widman flap procedure) at the baseline visit.

The subjects were randomly divided by computer-

Fig. 1 Modified Widman flap procedure (first incision in buccal surface).

Fig. 3 Third incision was made using Orban knife.

Fig. 2 Second incision was made and the flap was reflected.

Fig. 4 The flap returned and sutured using interrupted sutures.

Fig. 5 Collection of GCF.

generated random codes into three groups ($n = 10$). Group 1 included patients who received NAC capsules (600 mg; Swanson Health Products Co., Fargo, ND, USA) once daily for 3 months as adjunctive to standard surgical periodontal treatment (modified Widman flap procedure). Follow-up for evaluation of clinical parameters and GCF collection was performed up to a period of 7 months. Group 2 included patients who received surgical periodontal treatment alone with a follow-up regimen similar to that for Group 1 (Figs. 1-4). Group 3 included periodontally healthy control subjects who met the following criteria for inclusion: probing depth ≤ 3 mm, no clinical attachment loss, and no bleeding on probing or radiographic evidence of bone loss. Clinical evaluation was performed and GCF was collected once. The clinical measurements included plaque index (PI) of Silness and Le (13), gingival index (GI) of Le and Silness (14), probing depth (PPD), and clinical attachment level (CAL).

Clinical evaluation

All subjects were screened by comprehensive periodontal examination. The periodontal status of the subjects was determined by measuring the PPD and CAL and by recording the GI and PI values. Clinical parameters were recorded by the same calibrated examiner using William's graduated periodontal probe. The measurements were rounded to the nearest millimeter. The

examiner underwent calibration exercises on six patients for the aforementioned periodontal parameters prior to the commencement of the study.

GCF sample collection

GCF samples were collected initially before treatment (baseline), and 1 month, 3 months, and 7 months after periodontal therapy in both study groups. The samples were obtained from two contralateral sites per patient. The sites were chosen according to PPD levels (≥ 5 mm) and radiographic evidence of bone loss. After proper isolation of the sampled site with cotton rolls (to prevent contamination with saliva), the tooth was air-dried and a sterile filter paper strip (1.5×20 mm²; Whatman; W&R Balston Ltd., Richmond, UK) was inserted gently into the sulci/pocket until mild resistance was felt, and left

Table 1 Demographic characteristics of the study population

Characteristic	Moderate-to-severe generalized chronic periodontitis		
	Modified Widman flap procedure with NAC	Modified Widman flap procedure without NAC	Healthy controls
No. of subjects	10	10	10
Age	38.8 ± 5.66	40.8 ± 4.68	39.6 ± 5.45
Sex (Male/Female)	5/6	5/4	4/6

in place for 1 min (Fig. 5). Strips contaminated by saliva or blood were excluded. After GCF collection, the strip was immediately placed in sterile Eppendorf vials. The vials were stored at -20°C before laboratory analysis. sRANKL was extracted from the filter paper by adding 200 μL phosphate-buffered saline to the Eppendorf tube. Then, the sample was vortexed, followed by centrifugation for 10 min at 3,000 g.

Analysis of sRANKL in GCF

The samples were analyzed using the enzyme-linked immunosorbent assay kit (ELISA; Life Science Inc., Wuhan, China) for determination of free human sRANKL according to manufacturer's guidelines. The microtiter plate provided in this kit had been pre-coated with a monoclonal antibody specific to sRANKL. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for sRANKL. The color change was measured spectrophotometrically at a wavelength of 450 ± 10 nm by an ELISA reader. The results of GCF RANKL assays were expressed as concentrations in nanograms per milliliter.

Statistical analysis

This study had a power of 90% to detect a mean difference of 1 mm in clinical attachment levels between the treatment and control groups for PD assuming approximately 30% change in primary outcomes, including PPD and CAL, within groups after treatment. The minimum required sample size was calculated as six for each group. To compensate for potential dropouts, 10 patients were initially recruited in each treatment group.

Statistical analysis was performed using a Statistical Package for Scientific Studies (SPSS) for Windows (version 16.0, SPSS, Chicago, IL, USA). Data for normality were examined by the Kolmogorov-Smirnov (KS) test. GI and PI data that did not achieve normality were analyzed using non-parametric methods such as Kruskal-Wallis with post Dunn test to compare the changes at different time intervals in relation to the baseline.

sRANKL levels observed at baseline and at the 3- and

7-month intervals did not achieve normality. Kruskal-Wallis with post Dunn test was used to compare the sRANKL levels in GCF between the control and chronic periodontitis groups at baseline and the 3- and 7-month intervals. However, sRANKL levels achieved normality after 1 month (second baseline) and were analyzed using one-way ANOVA with post Dunnett tests to compare changes between baseline and 1 month interval in the treatment groups.

PPD, CAL, and sRANKL level data were analyzed using parametric methods; one-way ANOVA with post Dunnett test was used to compare and correlate changes in the treatment groups at different time intervals in relation to baseline. Intergroup differences were analyzed by Student's unpaired *t*-test for independent samples. Clinical parameters as well as the sRANKL levels in GCF are expressed as mean $\pm$ standard deviation. Pearson's correlation coefficient was used to determine significant correlations between the sRANKL levels in GCF and the different clinical parameters. A *P* value ≤ 0.05 was considered statistically significant.

Results

No adverse effects were observed with the systemic use of NAC. The demographic and baseline clinical parameters were similar across all study groups, as shown in Table 1.

A comparison of clinical parameters between the surgical group with NAC (S-NAC) and the surgical group without NAC (S-nonNAC) at different time intervals is presented in Table 2. Comparisons between mean difference values for PPD and CAL between the surgical groups with and without NAC from second baseline (1 month) are illustrated in Table 3. Surgical treatment produced a significant reduction in PPD in both groups at 2 and 6 months after treatment when compared to the second baseline (1 month). However, the administration of NAC in conjunction with surgical treatment produced a significantly higher reduction in PPD 2 months after treatment when compared to surgical treatment alone.

A comparison of the sRANKL levels in GCF (ng/mL) between the surgical treatment groups with and without NAC and the control group is shown in Table 4. There

Table 2 Comparison of the clinical parameters between S-NAC and S-non NAC at different time intervals

Period	S-NAC		S-nonNAC	
	Mean	SD	Mean	SD
Plaque index (PI)				
Baseline	2.25	0.46	2	0.83
1 month ^b	0.5	0.54	0.88	0.64
3 months	0.38	0.52	0.57	0.54
7 months	0.5	0.55	0.67	0.52
Gingival index (GI)				
Baseline	2.38	0.52	2.43	0.54
1 month ^b	1.13	0.35	1.13	0.35
3 months	0.5*	0.54	0.43*	0.54
7 months	0.5*	0.55	0.83	0.41
Probing pocket depth (PPD)				
Baseline	5.88	0.84	6.05	1.07
1 month ^b	5.13	0.35	5.38	0.74
3 months	2.88*, ⁺⁺	0.99	3.71*	0.95
7 months	2.17*	0.98	2.67*	1.51
Clinical attachments level (CAL)				
Baseline	5.13	1.25	6.63	1.06
1 month ^b	4.5	1.07	5.75	0.89
3 months	3.88	1.25	4.86	1.57
7 months	3*	0.89	4*	1.67

⁺⁺: Statistically significant between the two groups at $P \leq 0.05$ (unpaired *t*-test), ^b: second baseline, *: statistically significant from second baseline at $P \leq 0.05$ (ANOVA with post Dunnett test).

Table 5 Correlation between clinical parameters and sRANKL levels in GCF in S-NAC

Variables	Correlation coefficient	<i>P</i> value
Test sites at baseline		
sRANKL & PPD	-0.63	0.10
sRANKL & CAL	-0.60	0.12
sRANKL & GI	0.14	0.74
sRANKL & PI	-0.29	0.48
Test sites at 1 month		
sRANKL & PPD	0.35	0.39
sRANKL & CAL	-0.03	0.94
sRANKL & GI	-0.24	0.58
sRANKL & PI	0.36	0.38
Test sites at 3 months		
sRANKL & PPD	-0.26	0.53
sRANKL & CAL	-0.28	0.51
sRANKL & GI	-0.59	0.12
sRANKL & PI	0.025	0.95
Test sites at 7 months		
sRANKL & PPD	-0.58	0.13
sRANKL & CAL	-0.61	0.11
sRANKL & GI	0.18	0.68
sRANKL & PI	0.37	0.37

No statistical significance at $P \leq 0.05$.

was a statistically significant difference in sRANKL levels between the S-NAC and S-nonNAC groups at the second baseline when compared to the first baseline level. However, there was no significant difference in sRANKL levels in the two treatment groups 2 and 6 months after surgical treatment when compared to the second base-

Table 3 Comparison of the mean difference values for PPD and CAL between S-NAC and S-nonNAC from second baseline (1 month)

	Period	S-NAC	S-nonNAC
PPD	3 months	2.25 ⁺⁺	1.66
	7 months	2.96	2.71
CAL	3 months	0.63	0.89
	7 months	1.5	1.75

⁺⁺: Statistically significant between the two groups at $P \leq 0.05$ (unpaired test).

Table 4 Comparison of sRANKL levels in GCF (ng/mL) between S-NAC and S-nonNAC and the control group

Period	S-NAC		S-nonNAC		Control	
	Mean	SD	Mean	SD	Mean	SD
Baseline	11.23 ^a	1.58	10.18 ^a	4.77		
1 month ^b	8.02*	1.27	8.80	3.79	6.88	0.97
3 months	8.18	2.60	7.77	1.69		
7 months	9.71 ^a	2.60	8.91 ^a	2.22		

*: Statistically significant at $P \leq 0.05$ from baseline (one-way ANOVA with post Dunnett test), ^a: statistically significant between control and surgical groups at different intervals at $P \leq 0.05$ (Kruskal-Wallis test), ^b: second baseline.

Table 6 Correlation between clinical parameters and sRANKL levels in GCF in S-nonNAC

Variables	Correlation coefficient	<i>P</i> value
Test sites at baseline		
sRANKL & PPD	0.25	0.63
sRANKL & CAL	-0.43	0.40
sRANKL & GI	0.61	0.20
sRANKL & PI	0.17	0.76
Test sites at 1 month		
sRANKL & PPD	-0.32	0.53
sRANKL & CAL	-0.65	0.16
sRANKL & GI	-0.49	0.32
sRANKL & PI	-0.31	0.55
Test sites at 3 months		
sRANKL & PPD	-0.37	0.47
sRANKL & CAL	-0.80	0.06
sRANKL & GI	0.21	0.69
sRANKL & PI	-0.20	0.71
Test sites at 7 months		
sRANKL & PPD	-0.01	0.98
sRANKL & CAL	-0.57	0.24
sRANKL & GI	0.53	0.28
sRANKL & PI	-0.08	0.1

No statistical significance at $P \leq 0.05$.

line. Furthermore, statistically significant differences in the sRANKL levels in GCF were consistently noted between the two treatment groups and the control group at different time intervals.

As seen in Table 5, no correlation between the clinical parameters of test sites and sRANKL levels in GCF were

observed in the S-NAC group at different time intervals. Similarly, no correlation was observed between the clinical parameters of test sites and the sRANKL levels in GCF in the S-nonNAC treatment group at different time intervals (Table 6).

Discussion

Progressive destruction of the tooth-supporting tissues in chronic periodontitis is the result of the host response to bacterial pathogens. Stimulation of phagocytic cells as a part of the host response by periodontopathogenic bacteria produces various molecular species, including ROS, via a respiratory burst. It has been reported that the excess production of ROS leads to oxidative damage of the gingival tissue and periodontal ligament, and is associated with osteoclastic bone resorption (15,16). ROS inhibits osteoblast differentiation and enhances osteoclastogenesis. ROS-induced bone resorption occurs directly or indirectly (via increased RANKL expression) through the modulation of kinases and transcription factor activities in both osteoclasts and osteoblasts (17).

The antioxidant has been described as a substance that, at low concentrations when compared to an oxidizable substrate, significantly delays or prevents the oxidation of the substrate (7). Evidence for antioxidant depletion in periodontitis has been shown in several studies (18,19). NAC is a thiol antioxidant and a precursor of L-cysteine amino acid that serves as a precursor of glutathione synthesis (8). Glutathione is an important antioxidant in gingival and periodontal tissues based on millimolar levels measured within the GCF in health (20). The present trial was a one of its kind interventional study used to evaluate the effects of NAC in patients with chronic periodontitis. The daily dose of NAC in the present study was 600 mg. NAC has been in clinical use for more than 30 years, primarily as a mucolytic agent suppressing oxidative stress (21,22). The oral administration of NAC at doses of up to 8,000 mg/day did not cause clinically significant adverse reactions in human studies (23).

In the present clinical study, NAC was administered systemically at a dose of 600 mg daily for 3 months. This dose represents the minimal dose recommended for the prevention of the exacerbation of chronic obstructive pulmonary disease, where NAC is prescribed for its antioxidant and anti-inflammatory effect (24,25). In the present study, sRANKL was investigated in GCF rather than saliva, serum, or plasma. GCF is considered as the most appropriate fluid to sample when investigating the periodontal status, because it passes through the tissues and thereby accumulates the biomarkers of various tissue events (26). In addition, Buduneli and Kinane (27)

mentioned in their systematic review that GCF can be obtained non-invasively, especially after treatment and with minimal discomfort to the patient; the GCF obtained in this manner consists of both locally synthesized and systemically derived molecules. In the present study, the sRANKL levels in GCF were significantly higher in the chronic periodontitis groups when compared to the healthy control group at baseline. This is in accordance with the results reported in the studies by Vernal et al. (28), Lu et al. (29), and Bostanci et al. (30).

The sRANKL levels in GCF at 1 month (second baseline), 2 months, and 6 months post-surgery were 8.02 ± 1.27 , 8.18 ± 2.60 , and 9.71 ± 2.60 ng/mL, respectively, in the surgical group with adjunctive use of NAC, and 8.80 ± 3.79 , 7.77 ± 1.69 , and 8.91 ± 2.22 ng/mL in the surgical group without adjunctive NAC. No statistically significant changes in sRANKL levels were noted at various time points in each group or between groups in the surgical groups alone or in those with NAC, except during the first month. This can be explained by the fact that NAC was supplemented only for a short duration. As per our knowledge, the present study is the first of its kind to evaluate the effect of surgical periodontal treatment with or without adjunctive NAC therapy for the management of periodontitis and its relation to the sRANKL levels in GCF.

The sites that received MWF with or without adjunctive NAC exhibited a significant reduction in PPD (2.96 or 2.71 mm, respectively) 6 months post-surgery. This was in accordance with the results of Becker et al. (31), who reported a PPD reduction of 2.2 mm with gain in CAL (0.56 mm) 1 year after MWF surgery. Moreover, Kaldahal et al. (32) demonstrated a mean PPD reduction of 1.6-2.95 mm for moderate and deep sites with a corresponding gain of CAL (0.92-1.88 mm) 12 months post-surgery.

The administration of NAC in conjunction with surgical periodontal treatment had a significant effect on the healing of periodontal tissues. The sites that received adjunctive NAC along with surgical treatment exhibited significant reduction in PPD 2 months post-surgery when compared to the non-NAC group. These changes were evident during the 3 months of drug administration and can be attributed to its known antioxidant and anti-inflammatory actions promoting the resolution of inflammation and repair. NAC administration did not affect GCF sRANKL levels beyond changes induced by periodontal treatment during the 3 months of drug administration. This can be attributed to the administration of a low dose of 600 mg (peak plasma concentration of 4.6 μ M) for a relatively short time (3 months). It has been

reported that NAC prevented alveolar bone loss in the rat model in a dose-dependent manner; a dose of 70 mg/kg produced a significantly larger effect than the body weight dose of 7 mg/kg (10). It has been postulated that the end result of the chronic administration of NAC is determined by both duration and dosage, and is due to the cumulative effect of low concentrations over long periods of time (24). In the management of chronic obstructive pulmonary disease, low concentrations of NAC (600 mg/day) exerted limited antioxidant and anti-inflammatory actions. On the other hand, administration of higher doses (≥ 600 mg/day) induced a significant drop in the exacerbation rate, which was related to the pronounced effects on oxidative stress and inflammation (23).

One of the significant findings in the present clinical study was that there was no significant correlation between sRANKL levels in GCF and clinical parameters at baseline or after treatment. However, conflicting results have been reported between studies regarding the correlation of sRANKL or RANKL/OPG ratio with disease severity; a negative correlation was reported by Mogi et al. (33), whereas positive correlations were reported by Bostanci et al. (30). On the other hand, no correlations were seen according to Lu et al. (29). In addition, there appears to be no correlation between RANKL or RANKL/OPG ratio in GCF and gingival inflammation (30). Thus, it has been postulated that RANKL or RANKL/OPG ratio shows some specificity for the occurrence of periodontal bone loss, and thereby, periodontitis, rather than constituting a conventional marker of periodontal inflammation (34).

Limitations of the study

Following are the limitations of the present study: NAC was used at a dose of 600 mg daily for 3 months, based on the minimal dose recommended for the prevention of exacerbation of chronic obstructive pulmonary disease; NAC was used for 3 months because no other study has proven the drug supplementation beyond 3 months. Nevertheless, it can be concluded that there was a significant reduction in probing pocket depth in the surgical group with adjunctive use of NAC when compared to the group with surgery alone. However, no statistically significant correlation was observed between the sRANKL levels in GCF with the adjunctive use of NAC. Furthermore, multicenter, long-term, randomized, double-blinded clinical trials with larger sample size for the outcomes of systemic administration of NAC are needed to assert or rebut the usefulness of this agent as a therapeutic agent for periodontitis.

Conflict of interest

None declared.

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