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Research Article

EVALUATION OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION ON SMOKERS AND NON-SMOKERS WITH PERIODONTITIS

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ABSTRACT

Objective: It's aimed to evaluate neutrophil extracellular trap (NET) in smoker and non-smoker periodontitis patients.

Materials and Methods: 21 subjects were included in this study and divided into three groups [non-smokers and periodontally healthy (S-P-), non-smokers with periodontitis (S-P+), smokers with periodontitis (S+P+)]. Clinical attachment level (CAL), probing depth (PD), plaque index (PI), and gingival index (GI) parameters were recorded. To induce NET formation the peripheral neutrophils were isolated from the subjects and were activated by phorbol 12-myristate 13-acetate (PMA). Active NET was measured with the fluorometer.

Results: PI, GI, PD and CAL values were found statistically significant low in the S-P- group (P=0.001; P=<0.001; P=0.003; P=<0.001, respectively). NET count values were the highest in the S+P+ group. There was statistically significant difference between S-P- group and S+P+ group was (P=0.003); it was not statistically significant between S-P+ and S+P+ groups (P>0.05). NET count with CAL has strong positive relation and with PI, GI, PD, has mild positive relation.

Conclusion: As a result, there was no statistically significant difference between S-P+ and S+P+ groups, even the S+P+ group NET count was found higher. Further researches are needed on the effect smoking on NET formation in patients with periodontitis.

Keywords: Periodontitis, Smoking, Neutrophil Extracullular Trap

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INTRODUCTION

Periodontitis is a chronic, multifactorial, inflammatory disease associated with dental biofilm and is characterized by progressive destruction in supporting structures of the teeth. Its main feature is the loss of periodontal supportive tissues, which is characterized by clinical attachment loss and alveolar bone loss radiographically in the presence of periodontal pocket and gingival bleeding (1). Periodontal destruction can progress rapidly in relation to systemic and environmental risk factors that can modify the host immune response (2).

Smoking is an independent risk factor for the onset and exacerbation of periodontal disease (3).In periodontal health, there is a balance between the bacterial dental plaque and the immune-inflammatory response; however, periodontal health is impaired in relation to disruption of the host response or balance between host and bacteria, researches have shown that there is a spectrum of inflammatory responses to dental plaque accumulation. The immune-inflammatory response of the host is essential to determine the pathogenesis of periodontitis in individuals (4). It is now known that a large proportion of tissue destruction occurs as a result of the host's immune-inflammatory response (5). Neutrophils are considered to be the first line of defense against infectious agent in periodontal tissues. However, the presence of neutrophils is not always protective (6). Neutrophil counts in inflammatory periodontal tissues are correlated with the severity of the disease and hyperactive neutrophils are thought to cause tissue damage (7). The presence of severe forms of periodontitis in all congenital conditions associated with damage to the number and function of neutrophils such as Chediak-Higashi syndrome, Papillon-Lefèvre syndrome, lazy leukocyte syndrome and leukocyte adhesion defect (8).

At the same time, hyperreactive peripheral neutrophil response associated with excessive reactive oxygen species (ROS) has been shown in chronic periodontitis (9). ROS is produced with the chain of reactions called oxidative burst in neutrophils (10). Functional NADPH oxidase is required for ROS production (11). It is thought that tissue damage in periodontitis is caused by prolonged release of neutrophilic enzymes and ROS produced in response to microbial dental plaque (10).

Cigarette causes direct or indirect production of excess ROS with many chemicals it contains, it stimulates ROS production during inflammation (12). In a study on individuals with periodontitis, ROS was found to be higher in inflamed regions (13). In previous studies conducted on smokers with non-smokers with chronic periodontitis, it was found that ROS level was higher in individuals with periodontitis and smokers compared to healthy controls and non-smokers (14,15). It is thought that ROS production increases with cigarettes and increased ROS production plays a role in the periodontal disease pathogenesis (16).

In 2004, Brinkmann discovered the extracellular defense mechanism of neutrophils (17). Neutrophil extracellular trap (NET) is produced by stimulated neutrophils by IL-8, PMA or lipopolysaccharide (LPS). The major structural component of NET is DNA including histones and antimicrobial proteins such as NE, MPO, cathepsin G, lysozyme (18). These structures bind to the Gr (+) and Gr (-) bacteria and trap the bacteria and kill them with antimicrobial proteins (19).The initiation of NET formation (NETosis) is the activation of the cell by ROS produced by NADPH oxidase (20-22). Fuchs et al. (2007) showed that NADPH oxidase activation

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and ROS production are the keys for NETosis (20). ROS act as the second messenger during the NET formation and causes the nuclear membrane to be separated and completely lost (19).

It is thought that increased NET formation plays a role in the pathogenesis of many inflammatory diseases and NET release in pathological conditions is associated with severe tissue damage (23,24). Similar to other inflammatory diseases, it has been proposed that ROS dependent NET production may increase in periodontal disease (24). In a study, the presence of NET in the inflamed areas of the gingiva has been shown in individuals with chronic periodontitis (25). Therefore, it was reported that periodontal tissue damage may be associated with increased NET production (26). In another study, NET has been detected in the dental biofilm. In addition, the bacterial species in dental biofilm has been shown to cause NET formation in neutrophils isolated from patients (27). In chronic inflammatory diseases such as periodontitis, it has been suggested that excessive or non-functional NET production, the ability of bacteria to escape from the trap, the presence of non-removed NET after inflammation may cause tissue damage (22,24). In addition to this, NET formation may increase due to smoking, as a result of which tissue damage and immune disorder may occur in smoking related diseases (25). Nicotine has been also shown to induce NET formation (26).

The role of NETs in the pathogenesis of inflammatory diseases is new in the literature. It has not been previously investigated how this defense mechanism of neutrophils has been affected by smoking in individuals with periodontitis. The aim of our study was to measure and compare the amounts of NET in smoker and non-smoker individuals with periodontitis and to determine the correlation of NET amount with clinical parameters. The hypothesis of our study was that NET formation in smokers with periodontitis is higher than in non-smokers periodontitis and healthy individuals.

MATERIALS AND METHODS

Participants were selected from the patients admitted to Kirikkale University Faculty of Dentistry between October 2018 and December 2018. The participants were informed about the purpose and method of the study and informed consent was obtained from the participants who agreed to participate in the study. It has been decided that our study is in compliance with the ethical principles specified in Kırıkkale University Clinical Research Ethics Committee Directive (24.10.2017; 19/02).

Twenty-one systemically healthy individuals aged between 30 and 60 years were included in the study.

The inclusion criteria were deep periodontal lesions or multiple tooth losses leading to the apical third of the root,5 mm or more CAL in interdental area, radiographic bone loss extending to the middle or apical third of the root, at least 5 teeth lost due to periodontitis, patients need complex treatment because of loss of chewing function, secondary occlusal trauma, severe crest defect for individuals with periodontitis; smoking 10 or more cigarettes a day for at least 10 years for smokers; never smoked for non-smokers. Exclusion criteria were to have systemic disease that may affect periodontal health (diabetes, quantitative and / or qualitative polymorphonuclear neutrophil defects, and other immune system disorders), use antibiotics in the last 3 months, have periodontal treatment in the last 6 months, pregnancy and lactation period.

Participants who met the inclusion criteria are divided into three groups:
Group: Non-smoker and periodontal healthy individuals (n=7) (S-P-)
Group: Non-smoker individuals with periodontitis (n=7) (S-P+)
Group: Individuals with smoking and periodontitis (n=7) (S+P+)

Periodontal examinations

Periodontal clinical examinations were performed in all subjects. PI, GI, PD and CAL were measured from all participants. PD and CAL from the six regions and PI and GI from four regions of each tooth were evaluated. During the periodontal measurements, William's probe was used (Hu-Friedy, Chicago, USA).

Peripheral venous blood collection from participants

After the completion of periodontal examinations, 5 ml peripheral venous blood sample was obtained from the vein in the antecubital fossa of all the participants by using a tube contains anticoagulant.

PMN isolation from peripheral venous blood samples

As the Sursal et al. (2018) indicated that PMN isolation performed by using 72, 63, 54 and 45% Percoll dilutions. PMNs were counted using a Neubauer chamber under light microscopy (Leica DM750, Germany) and then reconstituted as $1x10^5$/100 µl in RPMI 1640 (Sigma).

Determination of PMN's viability and purity

Trypan blue solution (Sigma-Aldrich) was used to determine the viability of PMN. Under the light microscope stained cells were evaluated as died. The percentage of dead cells in the total number of cells was calculated as%.

To detect the neutrophil purity, the PMN samples were smeared on the slides. The slides were stained with Diff-Quick staining (Bio Optica) according to manufacturer protocol. Neutrophils were observed as cells with pale pink cytoplasm and dark blue polymorph nucleus under the light microscope. All neutrophils were counted in ten microscopic areas randomly selected. The proportion of neutrophils was compared with all polymorphonuclear cells counted.

PMA activation of neutrophils and quantitative analysis of NET

PMN dilutions (10^5/100 µl) were placed in sterile reaction tubes. PMA (25 nM, Fluorochem) was added to the reaction tubes. The tubes were incubated in the incubator containing 5% CO_2 (NuveMN120, Turkey) for 60 min at 37°C. At the end of the incubation, Micrococcal nuclease (5 U, NEB) was added to the reaction tubes and incubation was continued for 15 minutes under the same conditions. At the end of this period, the tubes were centrifuged at 300xg, 17°C for 7 min (Thermo Scientific 16SR). The supernatants were transferred into wells of a 96 well flat bottom black polystyrene plate (Nunc Thermo Scientific), each sample was tested in duplicate. Sytox orange dye (5 µM, Invitrogen) was added to the wells and they were incubated for 5 minutes in the dark at room temperature. The fluorescence level of the extracellular DNA was measured with a

fluorometer (Fluoroscan Ascent FL Thermo Scientific, USA) (355 nm excitation /460 nm emission). PMN which was not exposed to any treatment was used as the negative control in the experiment.

Statistical analysis

For descriptive statistics, the continuous variables were summarized with arithmetic mean and standard deviation or median and minimum - maximum values, and the categorical variables were summarized with frequencies and percentages. Mann-Whitney U or independent t test with Bonferroni correction test was used to compare two independent groups and Crustal Wallis test was used to examine the difference between three or more independent groups. In the case of significant differences, pairwise comparison tests were used to determine the different group(s). Wilcoxon test was used to determine the difference between two dependent groups. Pearson correlation coefficient was used to determine whether there is a significant relationship between numerical variables. $p<0.05$ was taken as significance level. The analyzes were performed using IBM SPSS v.21. In this study, the adequacy of the number of samples was calculated in G Power computer program where a total of 21 subjects were sufficient (Seven individuals in each group) with = 0.05, power = 0.85, and effect size = 0.8.

RESULTS

Study population, demographic data and clinical periodontal parameters

A total of 21 individuals, 9 women and 12 men [(S-P-) 7 individuals; (S-P+) 7 individuals; (S+P+) 7 individuals] were included the study. Analysis showed that sex was homogeneous in groups (p=0.853). Statistically significant difference was determined in age between the groups (p=0.045). When the groups were examined, it was seen that there was a significant difference between the ages of the S-P- and S-P + groups (p=0.040). Also there were significant differences between the groups in terms of PI, GI, PD and CAL (p=0.001; p≤0.001; p=0.003; p≤0.001, respectively). In S-P-group PI, GI, PD and CAL values were significantly lower than the other groups (Table 1).

PMN counts

There was a difference in cell numbers between S-P- and S + P + groups. However, this difference was statistically significant borderline (p=0.05; Table 2). The neutrophil purity was higher than 97% of the total cells. PMN viability was calculated as 98%.

Quantitative analysis of NET

When the amount of NET formed on PMA-activated and non-treatment (the negative control) neutrophils were compared, there was a statistically significant difference between the groups in terms of negative control values (p=0.009). It was seen that the group that makes difference is S-P- group. The negative control values in the S-P- group were lower. A significant difference was found between the groups in terms of positive control (NET) values (p= 0.003). It was seen that the group that makes difference is S-P- group. NET was lower in S-P group. S-P-group differed from both S-P + and S+P+ groups (p=0.013 and p=0.006). When the

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S-P+ and S+P+ groups were compared in terms of the amount of NET, no statistically significant difference was found ($p>0.05$; Table 3). When individuals with periodontitis (S-P+, S+P+) and healthy (S-P-) controls were compared, a significant difference was found between periodontitis and healthy individuals ($p<0.001$; Table 4).

Table 1. Age (median, min-max) and sex distribution of individuals (percentage) and comparison of clinical variables

		S-P- (n=7)	S-P+ (n=7)	S+P+ (n=7)	p value
Sex	**Female**	4 (57.1)	2 (28.6)	3 (42.9)	0.853
	Male	3 (42.9)	5 (71.4)	4 (57.1)	
Age		31 [30-53]^{a}	43 [36-60]^{b}	38 [30-54]^{a,b}	0.040
PI		0.32 [0.18–0.45]^{a}	1.98 [0.96–2.50]^{b}	2.35 [1.23-2.80]^{b}	0.001
GI		0.31 [0 – 0.37]^{a}	2.15 [1.14-3]^{b}	1.46 [1-1.76]^{b}	<0.001
PD		2.15 [1.36-2.31]^{a}	2.96 [1.54-3.83]^{b}	3.3 [2.60- 4.66]^{b}	0.003
CAL		0 [0 – 0]^{a}	4.12 [3.20-4.92]^{b}	4.85 [3.83-6.12]^{b}	<0.001

^{a,b}: The differences in those two groups; PI: Plaque Index, GI: Gingival Index, PD: Probing Depth, CAL: Clinical Attach-ment Level

Table 2. Comparison of PMN cell counts in groups

	S-P- (n=7)	S-P+ (n=7)	S+P+ (n=7)	p value
Cell Count (10^3)	1280 [700-1800]^{a}	1400 [600-3000]^{a,b}	2100 [1000-4000]^{b}	0.050

^{a,b}: The differences in those two groups

Table 3. NET amounts in groups (median, min-max)

	S-P- (n=7)	S-P+ (n=7)	S+P+ (n=7)	p value
Negative Control	1.742 [0-2.70]^{a}	2.509 [2.13- 2.82]^{b}	2.749 [2.10-2.78]^{b}	0.009
Positive Control (NET)	1.037 [0.78-1.99]^{a}	2.409 [2.03-2.78]^{b}	2.693 [1.43-2.95]^{b}	0.003
p value	0,866	0,310	0,271	

^{a,b}: The differences in those two groups

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Table 4. NET amounts in periodontitis and healthy individuals (median, min-max)

	Healthy Individuals (S-P-)	Individuals with Periodontitis [(S-P+) (S+P+)]	p value
NET	1.037 [0.781-1.99][a]	2.437 [1.43-2.95][b]	0.001

[a,b]: The differences in those two groups

The correlation of NET amounts and clinical parameters

It was observed that there was a statistically significant and mild positive correlation between NET amounts and the PI value (67%; p=0.001), the GI value (69%; p≤0.001), the PD value (53.3%; p=0.013). A statistically significant and strong positive correlation was found between NET amounts the CAL value (79.2%; p≤0.001; Table 5).

Table 5. The correlation of NET amounts with clinical parameters

		Pearson Correlation Coefficient	
		r	**p value**
NET	**PI**	0.67	0.001*
	GI	0.696	<0.001*
	PD	0.533	0.013*
	CAL	0.792	<0.001*

*Positive correlation

PMA activation of neutrophils

In each of the three groups, a portion of the PMN isolated was activated by PMA and a portion was left as a negative control without treatment. There was no statistically significant difference in PMA activation (p= 0.866; p= 0.310; p= 0.271, respectively; Table 3).

DISCUSSION

In our study, age, S-P+ and S+P+ groups were higher in the S-P+ group. However, only the difference between the S-P-group was statistically significant. Epidemiological studies reported that the prevalence and severity of periodontal disease increase with increasing age (31-33). It has been reported that periodontal disease starts earlier in smokers (34, 35).

According to our results, all clinical periodontal parameters were significantly higher in S-P+ and S+P+ groups than in S-P-group. PI, PD and CAL values were higher and GI values were lower in S+P+ group than

S-P+ group. However, these differences were not statistically significant. Generally, in smoker subjects, bleeding on probing values are reported at lower despite high PI. In addition, PD and CAL were higher than non-smokers (36, 37).

Studies have shown that the number of peripheral neutrophils in individuals with periodontitis is higher compared to healthy controls (38). The number of neutrophils was higher in smokers compared to non-smokers (39,40). In accordance with the current literature, the PMN count was the highest in S+P+ group and the least in the S-P-group. In terms of the PMN count, the difference between these two groups was statistically significant. However, there was no statistically significant difference in the number of PMN between S-P+ and S+P+ groups. Several factors have been reported in the literature that affects the number of PMNs except periodontitis and smoking. Male gender, high body mass index, high blood pressure, hyperlipidemia and diabetes were associated with increased PMN numbers (40). Although systemically healthy individuals were included in our study, no information was obtained about the body mass index or presence of hyperlipidemia. It can be thought that these factors may be effective in the results.

PMA causes activation of NADPH oxidase and ROS production and it has been reported to induce NET formation in neutrophils (17, 2041). Based on this information, $10x10^5$ PMN/ml neutrophils were activated with PMA at 37°C and 5% CO_2 and after 60 minutes NET was measured quantitatively in order to observe the effect of smoking on the amount of NET in our study.

It is thought that increased NET formation plays a role in the pathogenesis of many inflammatory diseases and it is associated with severe tissue damage. Similar to other inflammatory diseases, it is indicated that ROS-dependent NET production may increase in periodontal disease (24). NET was visualized with TEM in the inflamed areas of the gingiva in biopsy specimens from individuals with chronic periodontitis and found in purulent exudate from individuals with gingivitis (27). The other study demonstrated the presence of neutrophil and NET in dental biofilms and GCF. It has shown that bacterial species in dental biofilm causes NET formation in neutrophils. However, there was no correlation between the NET amount and clinical parameters in dental plaque (29).

According to the results of our study, there were mild positive correlations between the amount of NET and clinical parameters such as PI, GI and PD and there was a high positive correlation with CAL. CAL is a very important clinical parameter used for the diagnosis of periodontal disease and reflects the severity of the disease (42). The high positive relationship between the amount of NET and CAL in individuals with periodontitis highlights the potential role of NET in periodontal tissue destruction. In the previous study, some bacteria in dental biofilm such as T.f, P.m, F.n, P.i and P.g which are capable of release deoxyribonuclease (DNase) and degrade NET structures, may cause prevent to determine the relationship between NET and clinical parameters (24, 43).

In our study, the amounts of NET in both periodontitis groups were statistically higher than the S-P-group. The observed difference was thought to be a result of increased ROS production in inflammatory diseases. This finding supports the literature (24).

Studies have shown that smoking increases neutrophil-induced ROS production (16,44). ROS levels were found to be higher in individuals with periodontitis compared to healthy controls and the highest in smokers with chronic periodontitis (14,15). It is thought that NET formation may increase due to smoking, as

a result of which tissue damage and immune disorder may occur in smoking related diseases (25). In a study to evaluate the effect of smoking on NET formation in COPD patients, it was observed that the amount of NET activated by PMA in PMNs isolated from mice exposed to cigarette smoke was higher than control (45). In addition, nicotine has been shown to induce NET formation (24). According to the results in our study, the amount of NET in the S+P+ group was higher compared to the S-P+ group and the S-P- group. Although the difference between S+P+ group and S-P-group was found statistically significant, the difference between S-P+ group was not significant. Lack of significant difference between the groups with periodontitis may be due to the fact that smoking status is obtained subjectively, and different doses of smokers were not included in the study. Furthermore, in the literature, there are studies reporting that smoking suppresses the neutrophil NADPH oxidase and the oxidative burst exposed to CSE in mice (46, 47) It has been found that PMA-activated NET production is inhibited in neutrophils exposed to CSE and thiocyanate (SCN-) in cigarette smoke (48). Despite all these studies, the effect of smoking on the amount of NET is not clearly explained (49).

According to our knowledge, this study is the first that evaluates the effect of smoking on the amount of NET in smokers with periodontitis.

Limitations of our study were to evaluate smoking status subjectively, not to classify smoking dose and not to determine ROS markers simultaneously while evaluating NET formation.

CONCLUSION

In summary, in both periodontitis groups, NET was significantly higher than the healthy group. In smokers with periodontitis, the amount of NET was higher than in individuals with non-smoker periodontitis. However, the difference was not statistically significant. There was a positive correlation between the amount of NET and clinical parameters and this relationship was highly positive with CAL. The increased amount of NET was thought to play a role in tissue damage in periodontitis.

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NA

Authorship contributions

Surgical and Medical Practices: G.Y.O., E.O., Concept: G.Y.O., E.O Design: G.Y.O., H.O., Data Collection or Processing: G.Y.O., H.O, K.Y. Analysis or Interpretation: G.Y.O., H.O, K.Y., M.B. G.Literature Search: G.Y.O., H.O, K.Y., Writing: G.Y.O., H.O.

Data availibity statement

The data that support the findings of this study are available on request from the corresponding author.

Declaration of competing interest

The authors state that they have no conflict of interests.

Ethics

It has been decided that our study is in compliance with the ethical principles specified in Kırıkkale University Clinical Research Ethics Committee Directive (24.10.2017; 19/02).

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