Stomatologija, Baltic Dental and Maxillofacial Journal, 7:48-51, 2005

The Response of Peripheral Blood Neutrophils Unstimulated and Stimulated by Prodigiosan to Smoking

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SUMMARY

Alterations of neutrophils function by tobacco products may play a central role in the pathogenesis of periodontal diseases and several smoking related systemic diseases. The aim of the study was to assess a response of peripheral blood neutrophils unstimulated and stimulated by prodigiosan to smoking.

Materials and methods. The study included 17 smoking men that addressed for treatment of various odontological problems to out patient department of Kaunas University Clinic. The subjects were 22-43 years of age and systemic diseases free. All subjects answered to questions about smoking habits. To assess the response of neutrofils (unstimulated and stimulated by prodigiosan) to smoking the chemiliuminescence and nitroblue tetrazolium test were used to measure their oxidative metabolism.

Results. After smoking both neutrophils unstimulated and stimulated with prodigiosan showed considerable extracellular chemiliuminescence responses, but the first one reached maximal value in 45 min, while the second one in 15 min after smoking. There was no total (intra and extracellular) chemiliuminescence response of neutrophils unstimulated and stimulated by prodigiosan to smoking. Neutrofils exposed to smoking showed a significant increase in their nitroblue tetrazolium reduction.

Conclusion. Both stimulated by prodigiosan and unstimulated peripheral blood neutrophils release reactive oxygen species extracellulary which may alter the pathogenic processes in periodontal and other systemic diseases.

Key words: neutrophils, smoking, prodigiosan, reactive oxygen species, chemiluminescence, nitroblue tetrazolium reduction test

INTRODUCTION

Adult periodontitis is a chronic inflammatory disease that destroys the supporting structures of the teeth [1]. The disease may progress for many years without significant discomfort for the patient [2]. The microbial flora associated with subgingival plaques in humans with periodontal disease is believed to cause an inflammatory response and destruction of the gingival tissues [3,4]. This flora consists mainly of Porphyromonas gingivalis [5] but also of other anaerobic cocci, Bacteroides sp., Fusobacterium sp., Capnocytophaga sp., Selenomonas sp. ir Treponema sp. [2].

Alterations of neutrophil functions by both chronic low levels of tobacco and by acute short-term higher levels of tobacco smoke, as encountered during the act of smoking, may play a role in the pathogenesis of periodontal diseases and several smoking related systemic diseases. Some studies show that cigarette smokers demonstrate increased reactive oxygen species release from neutrophils [6,7,8,9]. Since both cigarette smoke and neutrophils are rich sources of oxidants, oxidation and inactivation of alfa 1-protease inhibitor in the lung fluid of smokers could lead to emphysema. Activated neutrophils from patients with stable emphysema produce more superoxide anion than those from healthy controls, especially in smoking subjects [10].

An accumulation of neutrophils in the gingival tissue may protect the host from potential pathogens [11]. The interaction between neutrophils and bacteria increases oxygen consumption and reactive oxygen species are produced which contribute to killing of the bacteria [12]. Reactive oxygen species deposited extracellulary, can however, destroy the surrounding tissues [11].

The aim of the study is to assess a response of peripheral blood neutrophils unstimulated and stimulated by prodigiosan to smoking.

MATERIAL AND METHODS

The study has been carried out in 17 smoking men that addressed for treatment of various odontological problems to out patient department of Kaunas University Clinic. The subjects were 22-43 years of age (age mean 33.06+4.48 yrs.) and systemic diseases free. The questionnaire about smoking (duration of smoking, number of cigarette smoked per day, type of cigarette (light, mild, strong) was used. We also calculated index of pack

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years (number of cigarettes smoked per day / 20 x duration of smoking in years).

To assess the response of neutrophils (unstimulated and stimulated by prodigiosan) to smoking the chemiliuminescence and nitroblue tetrazolium (NBT) reduction test were used to measure neutrophils oxidative metabolism.

Chemiluminescence assay. The neutrophils respiratory burst activity was determined by luminal/lucigenin-amplified chemiluminescence [13].

Ten milliliters of venous blood were collected from the subjects by venipuncture and anticoagulated with heparin (20 u/ml). The test tubes with blood were positioned at the angle of 45 degrees and kept for 1 h at 37°C. Then the supernatant plasma, rich in leukocytes, was aspirated, diluted with Hans balanced salt solution until 5 ml. and portions of 2 ml were taken to the tubes.

The intensity of spontaneous chemiluminescence of unstimulated neutrophils to an amplifier (lucigenin/ luminol) was measured after adding to plasma 0.1 ml of an amplifier (concentration 50 μM) in 3, 15, 30, 45, 60 min. before smoking and at the same time intervals after smoking.

Stimulation of neutrophils was made with 0.1 ml of 0.005% prodigiozan. Chemiluminescence of stimulated neutrophils to everyone above mentioned amplifier was measured in 3, 15, 30, 45, 60 min. before smoking and at the same time intervals after smoking.

Neutrophils make a major contribution to the total chemiluminescence response of whole blood or isolated cell suspensions. The intensity of chemiluminescence depends linearly on the amount of neutrophils in cell suspensions [14]. Therefore, the chemiluminescence intensity induced by neutrophils I(N) can be calculated from the chemiluminescence intensity of the total leukocyte fraction I(L) using equation: $I(N) = I(L) \times 100\ V/vcn$, where v is the cells suspension volume (ml), V is volume of the tube (ml), c is leukocyte concentration and n is the proportion of neutrophil (%) [13].

Chemiluminescence assay measuring the light intensity by a liquid scintillation counter Delta 300 (Model 6891, Trecor Europe BV Analytic Division) at the Department of Biochemistry of Kaunas University of Medicine.

The respiratory burst in normal neutrophils is associated with the generation of light energy or chemiluminescence which is dependent on the production of superoxide anion. Lucigenin dependent chemiluminescence shows oxygen radicals released extracellularly. Luminol dependent chemiluminescence presents total (intracellular plus extracellular) production of oxygen radicals.

The nitroblue tetrazolium (NBT) reduction test. NBT reduction test performed by Nagahata et al. [15].

Twenty milliliters of venous blood were collected from the subjects by venipuncture and anticoagulated with heparin (20u/ml). The test tubes with blood were positioned at the angle of 45 degrees and kept for 1 h at 37° C. Then the supernatant plasma, rich in leukocytes, was aspirated, diluted with Hans balanced salt solution until 5 ml and portions of 2 ml were taken to the tubes. Every 3.2 ml out of 9.6 ml of blood were put to the tubes where concentration of NBT was 116 μM. The control tube contained 0.4 ml of phosphate buffer.

The tubes were incubated at 37°C for 20 min in an incubator and centrifuged for 15 min. The samples for spectrophotometry were prepared by Talstad et al. [16]. The optical density of the solution was measured in spec-

Table 1. The characteristics of smoking

Index	N	Median	Mean	SD	Min	Max
Number of cigarettes /d	17	20	17.94	3.98	10	25
Type of cigarette*	17	2	2.06	0.75	1	3
Duration of smoking (yrs.)	17	16	14.41	4.49	6	23
Pack years	17	15	13.09	5.35	5	23

* Average of cigarette type, where 1 was "light", 2 was "mild" and 3 was "strong"

Table 2. Nitroblue tetrazolium reduction of neutrophils exposed and unexposed to smoking

Index	N	Median	Mean	SD	95% CI
Unexposed neutrophils	15	50	47.47	8.14	42.96-51.97
Exposed neutrophils	15	52	54.07*	10.07	48.49-59.64

*$p<0.05$

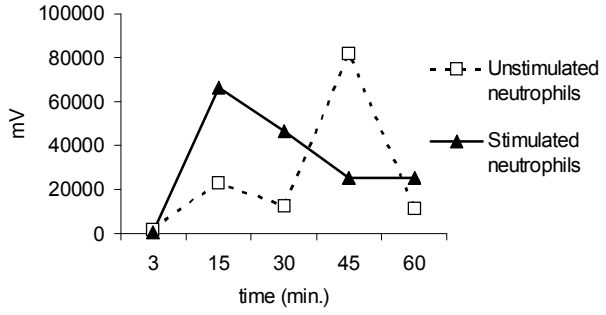

Fig. 1. . The neutrophil unstimulated and stimulated by prodigiosan extracellular chemiliuminescence response induced by smoking

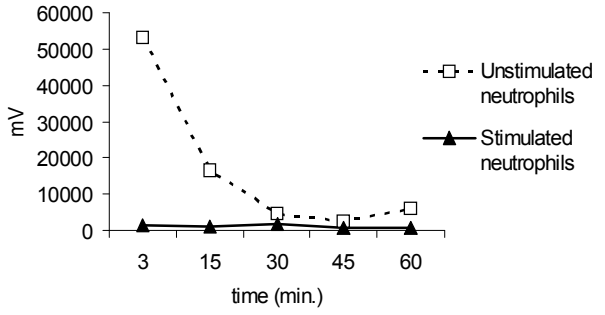

Fig. 2. The neutrophil unstimulated and stimulated by prodigiosan total (intra and extracellular) chemiliuminescence response induced

trophotometer CF-26, at 570 mm wave length.

The oxidative metabolism of the neutrophils was measured as the reduction of yellow-colored nitroblue tetrazolium to the dark blue formazan by the superoxide anion produced by the neutrophils [17].

Reagents. Lucigenin, luminol, Dulbecco phosphate buffer and Hans balanced salt solution obtained from Sigma Chemical Co Company (USA).

Statistical analysis. Mean values, median, standard deviation, 95% confidence intervals; minimal and maximal values of quantitative data are presented in the article. The Student's t test was used in tests with differences between arithmetic mean values. The level of significance was set at 5%. Statistical analysis was made with statistical package STATA 7.

RESULTS

Some characteristics of smoking are presented in Table 1. The average cigarette smoked per day was 17.94. The mean value of type of cigarettes was 2.06, i.e. mild cigarettes were mainly used by smokers. Average of smoking duration was 14.41 yrs. This index was very close to 13.09 pack years.

The results of the chemiluminescence are presented in Figures 1 and 2. After smoking both neutrophils unstimulated and stimulated with prodigiosan showed considerable extracellular chemiliuminescence responses, but the first one reached maximal value in 45 min, while the second one in 15 min after smoking. There was no total (intra and extracellular) chemiliuminescence response of neutrophils unstimulated and stimulated by prodigiosan to smoking.

The oxidative metabolism of unstimulated neutrophils was measured by NBT reduction test. The mean values are given in Table 2.

Neutrophils exposed to smoking showed a significant increase in their nitroblue tetrazolium reduction compare to that before smoking (respectively, 54.07 and 47.47, $p<0.05$).

DISCUSSION

Evidence from different studies in various populations demonstrates that adult smokers are approximately three times as likely as non-smokers to have periodontitis. Tobacco induced alterations in microbial and host factors contribute to these deleterious effects of smoking on the periodontium [18]. Recent studies indicate that one potential mechanism is that tobacco use exacerbates periodontal disease because it alters the immune response to periodontal pathogens [19].

Neutrophils are the first line of defense against bacterial infection, and although smokers have significantly higher numbers of neutrophils in the peripheral circulation,, their function is impaired. [20]. Neutrophils from smokers have shown decreased chemotaxis [21], phagocytosis [22, 23] and adherence [22].

Our data showed that after smoking neutrophils unstimulated and stimulated by prodogiosan released superoxide anion extracellulary. That was demonstrated by positive extracellular lucigenin dependent chemiluminescence responses during the first hour and a significant increase in neutrophil NTB reduction after smoking. We also found no intracellular chemiluminescence responses to smoking in 1 hour. According to Ryder et al. [8] unstimulated neutrophils exposed to smoke released more reactive oxygen species. Similar results were obtained by other authors [9,24]. However, the data were not consistent. Kalra et al. [6] reported luminol dependent chemiluminescence response to smoking that showed a significant increase in the oxygen-derived free radicals in smokers. On the other hand, Zhang [25] found that both lucigenin and luminol dependent chemiluminescence a concentration dependent inhibition response to cigarette-smoke tar.

In spite of some contradictions, which could appear because of some methodological differences, smoke exposure of unstimulated neutrophils has been shown to elevate the oxidative burst, which could enhance tissue destruction through direct and indirect toxic effects [20].

CONCLUSION

Both stimulated by prodigiosan and unstimulated peripheral blood neutrophils release reactive oxygen species extracellulary which may alter the pathogenic processes in periodontal and other systemic diseases.

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Received: 20 04 2005
Accepted for publishing: 20 06 2005