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The reduction of nitroblue tetrazolium by total blood in periodontitis patients and the aged

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SUMMARY

Experimental data have demonstrated that impaired antioxidant capacity is associated with increased levels of reactive oxygen species (ROS) production. ROS can cause inactivation of critical enzymes and induce denaturation that renders proteins nonfunctional. Total blood is continuously exposed to oxidative stress. The aim of the current study was to investigate the reduction of nitroblue tetrazolium (NBT) by the total blood in persons with periodontitis and aged subjects.

Materials and methods. The reduction of NBT in the total blood was measured according to a previously described procedure (Demehin et al., 2001), with modifications. Eight mL of venous blood were taken by venipuncture in the morning before meals and in portions of 2 mL placed into 4 plastic test tubes. Blood clotting was controlled with heparin (20 units/mL). Then the test tubes with blood were placed in the thermostat at the temperature of 37°C and kept for 5 min. Next, NBT, the final concentration of which ranged to 1×10^{-4}, was added to the blood in the test tubes, which were kept for 20 min at the temperature of 37°C. On completion of incubation, the tubes were centrifuged for 5 min at 2000×g to sediment any cells. The supernatant was decanted into fresh test tubes, and the absorbance of NBT in the samples was measured spectrophotometrically at wave length of 570 nm at 25°C.

Results. Total blood antioxidant capacity in the patients with periodontitis and aged subjects was significantly lower ($p<0.01$ and $p<0.05$, respectively) than in the control subjects.

In conclusion, the present study raise the necessity to search for causative agents of enhanced oxidative stress in patients with periodontitis and aged subjects and thus find the methods to diminish them.

Key words: reactive oxygen species, nitroblue tetrazolium.

INTRODUCTION

Oxidative stress induced by reactive oxygen species (ROS), such as superoxide anion ($O\bullet_2^-$), hydroxyl radical (OH•) and a range of lipid peroxyl radicals produced in vascular cells is involved in the pathogenesis of a wide range of disorders [1, 2, 3], including periodontal diseases [4, 5]. Elevated plasma endotoxin and tumor necrosis factor-alpha activity were also observed in ischemic heart disease [6] and periodontitis [7] patients suggesting a significant pathophysiological relevance of the circulating blood as potent stimulus for systemic immune activation [6].

Furthermore, evidence in the literature suggests that oxidative stress induced by circulating blood is associated with an underlying systemic inflammatory response. Emerging data also increasingly support the notion that oxidative stress may a possible cause of blood-induced inflammation through the activation of polymorphonuclear cells and enhanced oxidant activity arising from the respiratory burst [6, 8].

Polymorphonuclear neutrophils (PMN) play an essential role in defending the host against invading microbial pathogens. The primary function of PMN in the innate immune response – to contain and kill invading microbial pathogens – is achieved through a series of rapid and coordinated responses culminating in phagocytosis and killing of the pathogens [9]. Neutrophils possess a potent antimicrobial arsenal that

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includes oxidants, proteinases, and antimicrobial peptides. PMN also produce prodigious quantities of reactive oxygen species through the activity of oxidant-generating systems such as phagocyte NADPH oxidase [10] and nitric oxide synthase [11, 12]. During ingestion (phagocytosis) of invading pathogens, antimicrobial compounds contained in granules and ROS generated at the phagosome membrane are released directly into the phagosome. This process compartmentalizes both the pathogen and the cytotoxic products and facilitates intracellular killing [13]. Additionally, neutrophils can release granule proteins in the extracellular environment, which degrade virulence factors and bind and kill bacteria in a contained microenvironment [14].

The phagocyte NADPH oxidase is dormant in resting cells and can be rapidly activated by a variety of soluble mediators (e.g., chemoattractant peptides and chemokines) and particulate stimuli (e.g., bacteria and immune complexes) that interact with cell-surface receptors [15]. Activated NADPH oxidase generates large quantities of ROS [16], especially in subjects with periodontitis [17]. Chemical or cellular antioxidants protect blood cells and tissues against oxidative stress by scavenging ROS generated from the inflammatory stimuli. Experimental data have demonstrated that impaired antioxidant capacity is associated with increased levels of ROS production [18].

This may suggest that the levels of ROS produced exceed the antioxidant capacity of molecules such as superoxide dismutase (SOD), glutathione peroxidase and catalase [19]. It is also possible that the function of these enzymes could be altered by ROS. The modifications of proteins by ROS can cause inactivation of critical enzymes and induce denaturation that renders proteins nonfunctional [20, 21].

The accumulation of oxidative stress modified proteins in blood may reflect upon deficiencies in one or more parameters of a complex delicate balance between the presence of prooxidants, antioxidants, repair, replacement and elimination of biologically damaged proteins [22, 23]. Total blood is continuously exposed to oxidative stress: reduced oxygen species generated by the oxidation-reduction of drugs or xenobiotics transported by blood and metabolism in various tissues and organs which release ROS into blood [15, 24].

In this study, we have used the reduction of nitroblue tetrazolium (NBT) in the total blood (TB) of patients with periodontitis, 70-80-year-old subjects, and persons with intact periodontal tissues without systemic disorders.

MATERIALS AND METHODS

Chemicals. NBT, phosphate buffered saline (PBS) and heparin were obtained from Sigma Chemical Co. (USA), plastic vials and other disposable pieces of plastic were obtained from Care Rot GmbH and CoKG.

Patient selection. For the study, 16 patients with chronic periodontitis were selected from a large number of individuals treated in the Department of Odontology at Kaunas University of Medicine Hospital, and fifteen 70-80-year-old subjects. Control group was composed of 22 healthy 18-30-year-old subjects. They were selected from students and personnel at the Department of Odontology. The study contingent consisted of the subjects without any systemic disorders who during two weeks prior to the start of the study did not use vitamin preparations.

Laboratory studies. The reduction of NBT was measured in the total blood according to a previously described procedure [25] with modifications. Eight mL of peripheral venous blood were taken in the morning before meals and in portions of 2 mL was taken into 4 plastic test tubes. Blood clotting was controlled with heparin (20 units/mL). Then the test tubes with blood were placed in a thermostat at the temperature of 37°C and kept for 5 min. Next, NBT, the final concentration of which ranged to 1×10^{-4}, was added to the blood in the test tubes, which were kept for 20 min at the temperature of 37°C. On completion of incubation, the tubes were centrifuged for 5 min at 2000×g to sediment any cells [26]. The supernatant was decanted into fresh test tubes, and the absorbance of NBT was measured in the samples using a spectrophotometer at wave length of 570 nm at 25ºC.

Statistical analysis. All the experiments were repeated three or more times. The data are expressed as the mean±SEM, if normally distributed, and statistical significance of the differences was determined using Student's *t* test.

RESULTS AND DISCUSSION

Currently, there are no gold standard methods for measuring antioxidant capacity or ROS-mediated tissue damage in humans. All the systems utilize different measurement indices, and the specificity of the biomarkers employed dictate the measurement obtained, which differs between assays and between different biological samples and their components [5].

Free radicals and other reactive species have extremely short half-lives *in vivo* (10^{-6}-10^{-9}s) and simply cannot be measured directly [27]. The majority of clinical studies employ biomarkers of oxidative stress or tissue

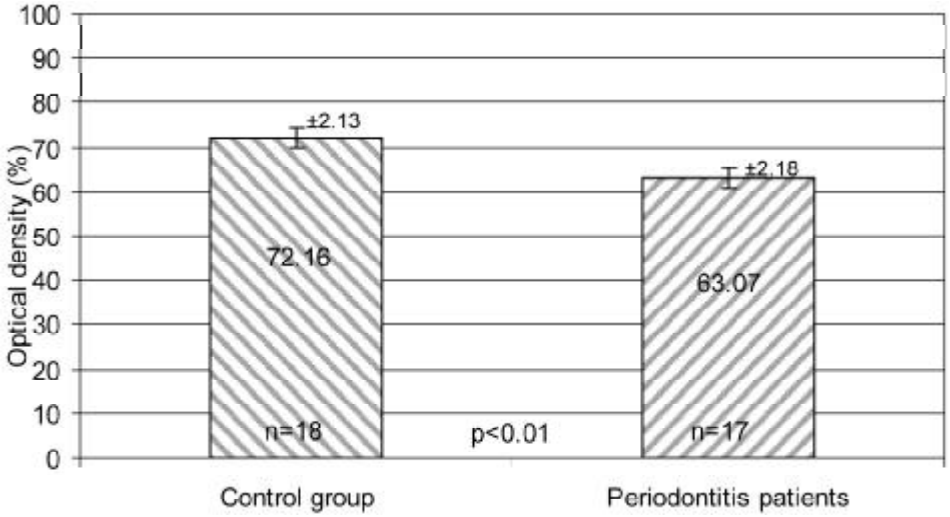

Fig. 1. Reduction of NTB by total blood of periodontitis patients. Results are presented as means. Error bars are SEM.

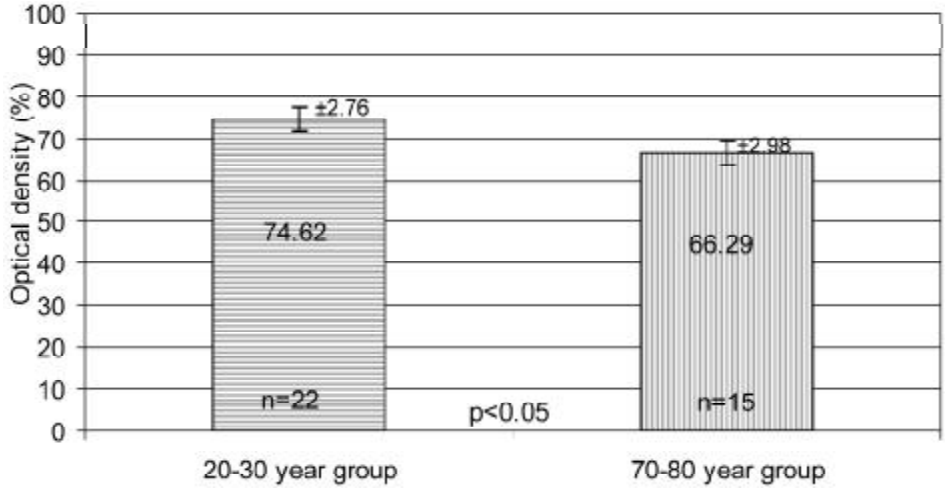

Fig. 2. Reduction of NTB by total blood of aged patients. Results are presented as means. Error bars are SEM.

damage to vital macromolecules, rather than spin traps. The main sources of biomarkers of ROS activity are:

- lipid peroxidation;
- protein/amino acid oxidation;
- carbohydrate damage;
- DNA damage.

All these assays are sufficiently sophisticated. Demehin et al. [25] suggested rather a simple method for assessment of total blood antioxidant capacity – the reduction of nitroblue tetrazolium test. Our study, using this test has shown (Fig. 1) that total blood antioxidant capacity in patients with periodontitis, was significantly lower ($p<0.01$) than in the healthy controls. We have not found in the medicobiological literature data on total blood antioxidant capacity in patients with periodontitis. And to our knowledge, only three studies were assigned for total antioxidant capacity of *serum/plasma* taken from periodontitis patients [28, 29, 30], one of them was done on dogs [31]. All the obtained data showed a significantly lower total antioxidant capacity of the serum and plasma samples taken from the periodontitis subjects [29, 30, 31]. Brock et al. [28] found that the reduced serum total antioxidant capacity in periodontitis did not completely reach significance, whereas differences in plasma levels did, which may reflect differences in serum and plasma preparation methods (serum is prepared at higher centrifugal forces and is more prone to oxidation) or the effects of clotting factor removal, or too small number of samples. Interestingly, *plasma* total antioxidant capacity in periodontitis subjects was significantly lower than that of *serum*.

Clinical experience and data of epidemiological studies [32] have shown high prevalence and incidence of periodontitis in the aged. The obtained by us study data concerning total antioxidant capacity of total blood have shown (Fig. 2) that they were reliably lowered ($p<0.05$) in 70-80-year-old subjects. Similar data were also found by us in the literature [25]. It has been explained by the findings that several red cell properties,

including cell volume [33] and deformability [34], change during the aging [25]. It has been suggested that many of these changes are associated with enhanced oxidative stress experienced by the red cells in elder subjects [25]. Increased oxidative stress is thought to be associated with increased active radical formation and/or decreased antioxidant levels [35].

CONCLUSIONS

In conclusion, the present study raises the necessity to reveal the causative agents resulting enhanced oxidative stress in periodontitis patients and the aged subjects and thus to develop the methods to reduce it.

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