ORIGINAL PAPER

THE INFLUENCE OF CHRONIC HYPER-HOMOCYSTEINEMIA ON CHARACTERISTICS OF PERIPHERAL NEUTROPHILS' PROGRAMMED DEATH IN LIPO-POLYSACCHARIDE-INDUCED PERIODONTITIS

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ABSTRACT

Introduction: Analysis of progression rate of periodontal disease indicates individual variability. It should be noted that destructive event in the alveolar bone has been linked to homocysteine (Hcys) metabolism; however, the issue has not yet been fully studied.

Objectives: To assess the peculiarities of the process of peripheral blood neutrophils programmed death in rats with lipo-polysaccharide (LPS)-induced periodontitis combined with chronic thiolactone hyper-homocysteinemia (HHcy).

Material and methods: 48 mature inbred white male rats were divided into four groups: control (n = 12), LPS-induced periodontitis (n = 12), chronic thiolactone HHcy (n = 12), and LPS-induced periodontitis combined with HHcy (n = 12). Analysis of peripheral blood neutrophil samples to determine all the studied parameters was performed on flow cytometer Epics XL (Beckman Coulter; USA).

Results: LPS-induced periodontitis in rats was accompanied by reactive oxygen species (ROS) hyper-production (by 87.9%, p = 0.001) and trans-membrane mitochondrial potential decrease by 73.3% (p = 0.001), which resulted in the distortion of integrity of the outer mitochondrial membrane, and the initiation of apoptotic events. Chronic thiolactone HHcy enhanced the initiation of the programmed death of neutrophils in case of LPS-induced periodontitis, which was confirmed by 71.4% significant prevalence of the apoptotic cells in animals with LPS-induced periodontitis combined with chronic thiolactone HHcy vs. rats with only LPS-induced periodontitis.

Conclusions: Excessive production of ROS and mitochondrial dysfunction caused by high serum Hcys level can be a crucial molecular mechanism that enhances programmed cell death in rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, which opens opportunities to improve pathogenetic therapy in patients with comorbid course of periodontal disease and chronic HHcy.

Key words: periodontitis, hyper-homocysteinemia, peripheral neutrophils, apoptosis.

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INTRODUCTION

The periodontium is a complex of tissues surrounding the tooth, which includes the tooth itself, periodontium, gum mucosa, and bone wall of the alveoli, which morpho-functionality can be treated as a single entity. Periodontal disease is one of the most spread pathologies of the oral cavity worldwide. It should be noted that loss of teeth due to periodontal disease is 5-10 times higher than the frequency of their extraction due to complications of caries [1].

According to the WHO data, incidence of periodontal disease correlates with the age of patients; in the 35-44 age group, the spread of this pathology is about 50%, and it reaches up to 65-78% in older population [2]. It should be mentioned that the prevalence of periodontal disease in developed and developing countries is different [3]. For instance, in European countries, the incidence of severe periodontitis in the cohort of 35-44 year old adults ranges from 5 to 20%, increasing to 40% in older population groups [4, 5]. At the same time, the frequency of periodontal disease among an adult population in Ukraine reaches 85-95% [6].

A characteristic feature of periodontitis is the chronic course of the disease, which leads to irreversible destruction of tissues that surround the tooth [7, 8]. In periodontitis pathogenesis, polymorphonuclear leukocytes (PMNs) mediate primary response against pathogenic micro-organisms, proliferating in the hosts' periodontal tissue. In activated PMNs, the production of reactive oxygen species (ROS) is sharply increased, which play the key role in the progression of periodontitis that results in the destruction of periodontium [9-12]. Moreover, progression of periodontitis is largely determined by the distortion of regulatory mechanisms of cell proliferation, differentiation, and programmed death in the gingival mucosa under the influence of numerous factors of aggression [13, 14]. Various gene expression patterns and apoptosis have been reported in other periodontal studies. Kebschull *et al.* compared transcriptional profiles of gingival tissue in cases of acute and chronic periodontitis. They found over-expression of the genes linked to signaling, apoptosis, and immune response in acute periodontitis, while in chronic disease, the over-expressed genes were linked to epithelial tissue integrity and metabolism [15].

Analysis of the progression rate of periodontal disease indicated individual variability. It should be noted that a destructive event in the alveolar bone has been linked to homocysteine (Hcys) metabolism; however, this issue has not yet been fully studied [16]. The role of Hcys in the progression, and the maintenance of periodontal diseases remains not fully understood. Probable mechanisms of a negative impact of hyperhomocysteinemia (HHcy) on the course of periodontitis may include activation of oxidative stress, which primarily causes damage to the endothelium as well as development of its' dysfunction [17, 18] and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is known to stimulate the synthesis of biologically active substances that in turn enhance leukocyte migration into the vessel wall, increasing leukocyte cyto-toxicity [19, 20].

OBJECTIVES

The study assessed the peculiarities of the process of peripheral blood neutrophils programmed death in rats with lipo-polysaccharide-induced periodontitis combined with chronic thiolactone HHcy.

MATERIAL AND METHODS

The present study included 48 mature inbred white male rats that were kept under standardized conditions at animal facility of I. Horbachevsky Ternopil National Medical University. Animal treatment and all experimental procedures were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (1986). Protocol of the experiment was approved by bioethics commission of I. Horbachevsky Ternopil National Medical University.

Rats were randomly divided into four groups (n = 12 per group): group 1 – control, group 2 – rats with a model of periodontitis. For two weeks, the animals were injected with 40 μl (1 mg/ml) of *E. coli* lipopolysaccharide (LPS) (Sigma-Aldrich, USA) into gingival tissues every other day [6]. Group 3 included rats with HHcy (n = 12). Homocysteine thiolactone was administered intra-gastrically (100 mg/kg of body weight in 1% solution of starch) once a day for 42 days [21]. Group 4 consisted of rats with a model of periodontitis combined with HHcy (n = 12). In animals of this group, chronic thiolactone HHcy was caused as described above. From the 29th day after the start of HHcy induction, animals were injected with homocysteine into the gum tissue with LPS for 14 days, according to the above-mentioned scheme in parallel with thiolactone.

Rats were sacrificed under deep thiopental-sodium anesthesia by cardiac puncture the day after the last LPS injection (group 2 and 4), or the day after the last homocysteine thiolactone administration (group 3). Whole blood was used for further studies.

To confirm the development of chronic HHcy, total serum Hcys level was determined by a solid phase enzyme immunoassay using Axis-Shield (Great Britain) reagent kit on a Multiskan FC analyzer (Thermo Scientific, Finland). Total serum Hcys level was expressed in μmol/l.

Isolation of the peripheral neutrophil population was carried out by centrifugation of whole blood at double density gradient 1.077 and 1.093 of Ficoll-Urografin [22]. Analysis of neutrophil samples was carried out on flow

cytometer Epics XL (Beckman Coulter; USA) in order to determine all investigated parameters. ROS (hydrogen peroxide, H_2O_2) production was assessed using 2.7-dichlorodihydrofluorescein diacetate (Sigma Aldrich; USA) [23]. Transmembrane mitochondrial potential (ΔΨm) was evaluated, using a MitoScreen kit of reagents (BD Pharmigen; USA) with fluorochrome JC-1 ($C_{25}H_{27}Cl_4IN_4$) as key reagent [24]. Neutrophils with signs of apoptosis/necrosis (Annexin V $(ANV)^+$-cells/propidium iodide $(PI)^+$-cells) were determined using FITC-labeled annexin V from ANNEXIN V FITC kit of reagents (Beckman Coulter; USA) [25].

Statistical analysis of the experimental data was performed using MS Office Excel (Microsoft Corp.; USA) and Statistica 7.0 (StatSoft Inc.; USA). Normality of variables' distribution was tested by Kolmogorov-Smirnov test. Since the variables were not normally distributed, three or more groups were compared using Kruskal-Wallis test. Mann-Whitney test with Bonferroni correction was subsequently applied in pair-wise group comparisons. Data within the groups were reported as median Me (interquartile range [IQR]). Results were considered statistically significant at a probability level p-value < 0.05. Additionally, Spearman's rank correlation coefficient (r) and its' p-value were calculated to find associations between the studied variables. For all tests, p-value < 0.05 was accepted as statistically significant.

RESULTS

The results of our study demonstrated that total serum Hcys level in case of LPS-induced periodontitis increased by 47.4% vs. the control group (Table 1). However, these changes were not statistically significant (p = 0.215). In animals with LPS-induced periodontitis combined with chronic thiolactone HHcy, the total serum Hcys level increased significantly by 3.8 times compared to controls, which was 2.6 times higher than the data obtained for only LPS-induced periodontitis. It should be noted that in animals with chronic thiolactone HHcy, Hcys level in blood serum demonstrated significant 3.4 times increase compared to the control group, and did not differ (p > 0.05) from the data obtained from rats with LPS-induced periodontitis combined with chronic thiolactone HHcy.

We found that ROS production by peripheral neutrophils of animals with only LPS-induced periodontitis significantly increased by 87.9% compared to the control group. In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index demonstrated a 2.8 times increase (p < 0.001) compared to controls, which was 50.5% (p = 0.008) higher than the indices for only LPS-induced periodontitis (Table 2). In animals with chronic thiolactone HHcy, ROS production by peripheral blood neutrophils increased by 54.8% (p = 0.007) compared to the control group, and did not differ (p > 0.05) from data of rats with only LPS-induced periodontitis.

It was found that in animals with only LPS-induced periodontitis, the number of peripheral blood neutrophils with reduced ΔΨm increased by 73.3% (p = 0.001) compared to controls (Table 2). In rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, this index showed a 2.5 times increase (p < 0.001) vs. controls, which was 42.3% (p = 0.001) higher than the data of only LPS-induced periodontitis. In animals with chronic thiolactone HHcy, the number of peripheral blood neutrophils with reduced ΔΨm increased by 40.0% vs. the control group. However, these changes were not statistically significant (p = 0.304).

While studying the number of peripheral blood neutrophils with signs of apoptosis, it has been determined that in animals with only LPS-induced periodontitis, this index showed a 2.1 times increase (p < 0.001) compared with the control group (Table 2). In animals with LPS-induced periodontitis combined with chronic thiolactone HHcy, there was a 3.5 times increase in number of ANV^+-cells, (p < 0.001) compared with controls, which was 71.4 % (p = 0.001) higher than the data for only LPS-induced periodontitis.

When studying the number of PI^+-cells, which characterize the intensity of necrotic processes, in animals with only LPS-induced periodontitis, a significant

TABLE 1. Level of homocysteine (Hcys) in blood serum of rats with LPS-induced periodontitis without comorbid pathology, and combined with chronic thiolactone hyperhomocysteinemia (HHcy) (Me [IQR])

Parameter	Experimental groups			
	1 – Control	2 – Periodontitis	3 – HHcy	4 – Periodontitis + HHcy
Hcys, μmol/l	7.70 (7.40; 8.15)	11.35 (10.15; 11.65)	26.39 (23.99; 29.63)	29.04 (26.45; 33.12)
	Kruskal-Wallis criterion: $H = 26.41$; $p < 0.001$*			
	$p_{1-2} = 0.215$ $p_{1-3} < 0.001$* $p_{1-4} < 0.001$*	$p_{2-3} = 0.035$* $p_{2-4} = 0.002$*	$p_{3-4} = 0.999$	–

*p_{1-2}, p_{1-3}, p_{1-4} – probability of differences between control and experimental groups; p_{2-3} – probability of differences between the group with periodontitis and the group with HHcy; p_{2-4} – probability of differences between the group with periodontitis and the group with periodontitis combined with HHcy; p_{3-4} – probability of differences between the group with HHcy and the group with periodontitis combined with HHcy; * – statistically significant results*

TABLE 2. ROS production, ΔΨm, and apoptosis/necrosis of peripheral blood neutrophils in rats with lipopolysaccharide-induced periodontitis without comorbid pathology, and combined with chronic thiolactone hyperhomocysteinemia (HHcy) (Me [IQR])

Parameter	Experimental groups			
	1 – Control	2 – Periodontitis	3 – HHcy	4 – Periodontitis + HHcy
	Suspension of blood neutrophils			
Increased ROS production cells count, %	16.05 (14.25; 19.40)	30.15 (27.50; 31.85)	24.85 (22.85; 26.60)	45.38 (41.13; 47.00)
	Kruskal-Wallis criterion: $H = 41.38$; $p < 0.001$*			
	$p_{1-2} = 0.001$* $p_{1-3} = 0.007$* $p_{1-4} < 0.001$*	$p_{2-3} = 0.272$ $p_{2-4} = 0.008$*	$p_{3-4} < 0.001$*	–
Decreased ΔΨm cells count, %	1.50 (1.20; 1.70)	2.60 (2.25; 2.80)	2.10 (1.80; 2.30)	3.70 (3.45; 3.95)
	Kruskal-Wallis criterion: $H = 37.42$; $p < 0.001$*			
	$p_{1-2} = 0.001$* $p_{1-3} = 0.304$ $p_{1-4} < 0.001$*	$p_{2-3} = 0.504$ $p_{2-4} = 0.001$*	$p_{3-4} < 0.001$*	–
ANV^+-cells count, %	2.20 (1.80; 2.45)	4.55 (4.25; 4.90)	3.40 (3.25; 3.60)	7.80 (7.45; 8.30)
	Kruskal-Wallis criterion: $H = 42.85$; $p < 0.001$*			
	$p_{1-2} < 0.001$* $p_{1-3} = 0.247$ $p_{1-4} < 0.001$*	$p_{2-3} = 0.182$ $p_{2-4} = 0.001$*	$p_{3-4} < 0.001$*	–
PI^+-cells count, %	1.35 (1.15; 1.65)	2.30 (2.15; 2.35)	1.90 (1.55; 2.00)	3.75 (3.65; 4.15)
	Kruskal-Wallis criterion: $H = 39.95$; $p < 0.001$*			
	$p_{1-2} < 0.001$* $p_{1-3} = 0.653$ $p_{1-4} < 0.001$*	$p_{2-3} = 0.116$ $p_{2-4} = 0.001$*	$p_{3-4} < 0.001$*	–

*p_{1-2} p_{1-3} p_{1-4} – probability of differences between the control and experimental groups; p_{2-3} – probability of differences between the group with periodontitis and the group with HHcy; p_{2-4} – probability of differences between the group with periodontitis and the group with periodontitis combined with HHcy; p_{3-4} – probability of differences between the group with HHcy and the group with periodontitis combined with HHcy; * – statistically significant results*

excess of the data obtained from the control group (by 70.4%) was found. Animals with LPS-induced periodontitis combined with chronic thiolactone HHcy had a 2.8 times higher number of PI^+-cells ($p < 0.001$) vs. the control group, which was 63.0% ($p = 0.001$) higher than the data for only LPS-induced periodontitis.

It should be noted that in animals with chronic thiolactone HHcy, the number of peripheral blood neutrophils both with signs of apoptosis and with signs of necrosis, did not significantly differ from the controls.

The analysis of the association between the amount of ANV^+-cells and the amount of cells with increased ROS releasing and reduced ΔΨm in animals with only LPS-induced periodontitis showed a strong direct correlative linkage between the amount of ANV^+-cells and the amount of cells with increased ROS releasing a moderate direct correlative linkage between the amount of ANV^+-cells and the amount of cells with reduced ΔΨm. In animals with LPS-induced periodontitis combined with chronic thiolactone HHcy, both of the above-mentioned correlative linkages had strong manifestations. As for the animals with chronic thiolactone HHcy, the correlative linkages between the amount of ANV^+-cells and the amount of cells with increased ROS releasing and reduced ΔΨm, turned out to be statistically improbable (Table 3).

The conducted correlative analysis between the amount of PI^+-cells and ANV^+-cells as well as the amount of cells with increased ROS releasing and reduced ΔΨm in rats with only LPS-induced periodontitis has determined only one significant direct moderate interaction between the number of PI^+-cells and the number of cells with increased ROS releasing (Table 3). A direct very strong association between the amount of PI^+-cells and the amount of neutrophils with a decreased ΔΨm as well as a direct strong linkage between the number of PI^+-cells and the number of neutrophils with an increased ROS production were determined in rats with LPS-induced periodontitis combined with chronic thiolactone HHcy.

Analyzing correlative linkages between Hcys levels in the blood serum and the count of PI^+-cells, the count of ANV^+-cells, neutrophils' count with an increased ROS production, and the number of neutrophils with a decreased ΔΨm, it should be noted that significant correlative linkages with all the studied indices were detected in rats with LPS-induced periodontitis combined with chronic thiolactone HHcy (Table 4).

TABLE 3. Correlative linkages between the count of peripheral blood neutrophils with signs of apoptosis/necrosis and the count of cells with increased ROS production and decreased ΔΨm in case of lipopolysaccharide-induced periodontitis without comorbid pathology and combined with chronic thiolactone hyperhomocysteinemia (HHcy) (r_{xy})

Parameter			
ANV^+-cells count, %	Increased ROS production cells count, %		
	Periodontitis	HHcy	Periodontitis + HHcy
	$r = 0.82; p = 0.001$*	$r = 0.29; p = 0.365$	$r = 0.83; p = 0.001$*
	Decreased ΔΨm cells count, %		
	Periodontitis	HHcy	Periodontitis + HHcy
	$r = 0.62; p = 0.031$*	$r = 0.25; p = 0.440$	$r = 0.77; p = 0.003$*
PI^+-cells count, %	Increased ROS production cells count, %		
	Periodontitis	HHcy	Periodontitis + HHcy
	$r = 0.58; p = 0.049$*	$r = 0.64; p = 0.024$*	$r = 0.76; p = 0.004$*
	Decreased ΔΨm cells count, %		
	Periodontitis	HHcy	Periodontitis + HHcy
	$r = 0.49; p = 0.110$	$r = 0.56; p = 0.060$	$r = 0.91^{\#}; p < 0.001$*

**Statistically significant results. #Significant differences of correlation coefficients; p < 0.05 (between the group of only periodontitis with the group of periodontitis combined with HHcy)*

TABLE 4. Correlative linkages between the level of homocysteine (Hcys) in blood serum and the count of peripheral blood neutrophils with increased ROS production, decreased ΔΨm, and signs of apoptosis/necrosis in case of lipopolysaccharide-induced periodontitis without comorbid pathology and combined with chronic thiolactone hyperhomocysteinemia (HHcy) (r_{xy})

Parameter		Experimental groups		
		Periodontitis	HHcy	Periodontitis + HHcy
Hcys, μmol/l	Increased ROS production cells count, %	$r = 0.76; p = 0.004$*	$r = 0.58; p = 0.047$*	$r = 0.82; p = 0.001$*
	Decreased ΔΨm cells count, %	$r = 0.62; p = 0.032$*	$r = 0.59; p = 0.044$*	$r = 0.65; p = 0.022$*
	ANV^+-cells count, %	$r = 0.56; p = 0.057$	$r = 0.79; p = 0.002$*	$r = 0.65; p = 0.022$*
	PI^+-cells count, %	$r = 0.51; p = 0.065$	$r = 0.69; p = 0.013$*	$r = 0.66; p = 0.020$*

**Statistically significant results*

DISCUSSION

Violation of the oral microbiota and formation of the biofilms have been determined as the leading etiological factors of periodontitis [26]. Of all PMNs in the periodontal pocket and gingival crevice, neutrophils comprise the largest fraction. Since neutrophils are also the largest leukocyte group in inflamed periodontal tissues, they act as a barrier preventing micro-organism ingress between dental plaque and junctional epithelium [27]. Neutrophils produce marked bactericidal effect; they neutralize pathogens by releasing free radicals (in so called 'respiratory burst'), and then absorbing them through phagocytosis [28]. Moreover, neutrophil cells contain an array of hydrolytic enzymes, including proteases, lipases, and deoxyribonucleases, in addition to compounds specifically targeting the bacterial cell wall, such as lysozyme and lactoferrin [29, 30]. Additionally, a new antibacterial defense mechanism has been discovered in recent years: neutrophil extra-cellular traps [31].

At the same time, excessive activation of neutrophils and ROS hyper-production as a reaction to periodontal pathogens can induce periodontium damage, and lead to the persistence of periodontitis [27]. Our results showed the significant increase of ROS generation by peripheral blood neutrophils, decrease of their ΔΨm as well as the increase of the number of cells with signs of apoptosis in case of LPS-induced periodontitis.

Our results are consistent with reports of other researchers, who showed an association between periodontal tissue damage, hyper-production of ROS, and apoptosis. Matthews *et al.* showed that neutrophils obtained from patients with chronic periodontitis demonstrated an increase of extra-cellular ROS release *in-vitro* without exogenous stimulation [32]. Other researchers showed that LPS from *Porphyromonas gingivalis* and hypoxia induce a NADPH oxidase 4-dependent increase in the release of H_2O_2 in periodontal ligament fibroblasts that may additionally facilitate the development and progression of periodontitis [33]. Oktay *et al.* observed

that rats, if compared with the control group, which were infected with the periodontal pathogens, manifested a 5-fold increase in oxidative stress index [34].

Parahonsky *et al.* examined 39 patients with periodontitis. They determined that while the levels cytochrome C (a marker of cell apoptosis) initially increase at the beginning of periodontics, they decrease afterward, as the disease progresses to its' final stages [35]. Sarkisov *et al.* examined 40 patients with periodontitis and found an increased level of annexin V in the oral fluid [36]. To determine the relationship between apoptosis and periodontium condition, the researchers performed a correlation analysis, which revealed a statistically significant linkage of moderate strength between the level of annexin V and the values of periodontal indices, which reflected the role of apoptosis intensification in the progression of inflammatory-destructive changes of the periodontium.

Therefore, the LPS-induced periodontitis in rats is accompanied by ROS over-production by peripheral blood neutrophils and the decrease of their ΔΨm, which consequently distorts the integrity of mitochondrial membrane and the launch of apoptotic death. The conducted correlative analysis indicates a probable linkage between the number of ANV^+-cells, number of cells with ROS over-production, and the number of neutrophils with a decreased ΔΨm, which indicates a mitochondrial pathway of apoptosis initiation.

Our results demonstrate that chronic thiolactone HHcy enhances the initiation of programmed cell death in case of periodontitis, which is confirmed by the significant prevalence of the number of peripheral blood neutrophils with the signs of apoptosis in rats with LPS-induced periodontitis combined with chronic HHcy, if compared with rats with LPS-induced periodontitis only. We suggest that ROS over-production and destruction of the mitochondrial inner membrane that occurs due to a decrease of trans-membrane potential, is one of the important signalling pathways of triggering programmed cell death, both in condition of LPS-induced periodontitis only, and in case of a comorbidity of LPS-induced periodontitis and chronic thiolactone HHcy.

Molecular mechanisms that underlie the pro-apoptotic effect of Hcys are not yet well-understood. Hcys-induced mitochondrial dysfunction can be a crucial molecular mechanism that mediates apoptosis. Hcys can cause endoplasmic reticulum stress and intra-cellular Ca^{2+} mobilization, which are followed by a subsequent development of programmed cell death [37]. Moreover, the consecutive leakage of cytochrome C from mitochondria as well as ROS activate the caspase 3 pathway, which leads to DNA fragmentation. Huang *et al.* observed that Hcys prompts apoptotic DNA damage mediated by increased intra-cellular H_2O_2 and activation of caspase 3 [38]. Kruman *et al.* have demonstrated that Hcys concentration influences the value and speed of apoptosis development in their experiment. For instance, a 250 μM concentration of Hcys caused the apoptosis of practically all hippocampal neurons in culture within 28 hours, while in the same experiment, a 0.5 μM concentration (which is an in-vivo norm) led to apoptosis of only 40% of neurons within 96-144 hours [39]. Bao *et al.* demonstrated Hcys-induced apoptosis in endothelial progenitor cells, which may be due to their pro-oxidative effects as well as because of an upregulation of p38MAPK protein expression and caspase-3 activity [40]. Alam *et al.* also showed that Hcys-mediated endothelial cells apoptosis is associated with caspase-8, cytochrome-*c* release and caspase-3 activation [41]. It has been revealed that Hcys induces mitochondrial apoptosis in SH-SY5Y cells [42] and in primary cultures of cyto- and syncytiotro-phoblastic cells [43]. Kim and Pae have conclusively demonstrated the dose-dependent toxicity of Hcys on the culture of cerebellar granule neurons. Hcys at a dose of more than 300 μM activates N-methyl-D-aspartate receptors within 16-22 hours, thus causing an increased production of ROS and initiation of programmed cell death [44]. In a study of Fang *et al.*, the viability of Neuro2a neuroblastoma cell line declined in a dose-dependent manner when incubated with Hcys; the cells underwent apoptosis within 48 hours. These effects were produced at a Hcys concentration of < 5 mM. The authors suggested that apoptotic process is initiated by nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as acylation [45]. Chen *et al.* described the Hcys-induced cell apoptosis in melanocytes via ROS and endoplasmic reticulum stress protein kinase RNA-like ER kinase (PERK) – eukaryotic translation initiation factor 2α (eIF2α) – C/ EBP homologous protein (CHOP) (PERK–eIF2α–CHOP signalling pathway) [46]. A number of mechanisms produced by Hcys resulting in adverse effects on mitochondrial and endoplasmic reticulum function were described by Zhang *et al.*, who investigated human umbilical vein endothelial cell (HUVEC) [47]. First, Hcys-induced mitochondrial dysfunction involved downregulated Bcl-2 expression, reduced mitochondrial transmembrane potential and amplified mitochondrial ROS, all of which resulted in elevated cytoplasmic cytochrome C, ROS, and caspase-3 levels, initiating HUVEC programmed death. Additionally, Hcys contributes to HUVEC apoptosis by modulating NF-kβ activation, as it triggers PERK, initiates eIF2α phosphorylation, and upregulates the expression of activating transcription factor 4 and CHOP. The endoplasmic reticulum stress is furthermore triggered by an increased cytoplasmic ROS, resulting in misfolded proteins.

STUDY LIMITATIONS

This study was performed on a small sample size; the results are therefore presented as preliminary. Further investigations are needed to explore the clinical implications of these findings.

CONCLUSIONS

Our study showed that chronic thiolactone HHcy enhances the apoptotic events in peripheral blood neutrophils of rats with LPS-induced periodontitis. Excessive production of ROS and mitochondrial dysfunction caused by high serum Hcys level can be a crucial molecular mechanism that enhances programmed cell death in rats with LPS-induced periodontitis combined with chronic thiolactone HHcy, which opens opportunities for improving of pathogenetic therapy in patients with comorbid course of periodontal disease and chronic HHcy.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1. Putri H, Sulijaya B, Hartomo BT, Suhartono AW, Auerkari EI. +17 C/G polymorphism in matrix metalloproteinase (MMP)-8 gene and its association with periodontitis. J Stoma 2020; 73: 154-158.
2. GBD 2017 Oral Disorders Collaborators; Bernabe E, Marcenes W, Hernandez CR, et al. Global, regional, and national levels and trends in burden of oral conditions from 1990 to 2017: a systematic analysis for the Global Burden of Disease 2017 study. J Dent Res 2020; 99: 362-373.
3. Germen M, Baser U, Lacin CC, Fıratlı E, İşsever H, Yalcin F. Periodontitis prevalence, severity, and risk factors: a comparison of the AAP/CDC case definition and the EFP/AAP classification. Int J Environ Res Public Health 2021; 18: 3459.
4. Peres MA, Macpherson LMD, Weyant RJ, et al. Oral diseases: a global public health challenge. Lancet 2019; 394: 249-260.
5. Balogun A, Taiwo J, Opeodu O, Adeyemi B, Kolude B. Impact of non-surgical periodontal therapy on the salivary levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) in patients with chronic periodontitis: a third world experience. Open Journal of Stomatology 2021; 11: 197-207.
6. Shcherba V, Krynytska I, Marushchak M, Korda M. Does thyroid dysfunction influence inflammatory mediators in experimental periodontitis? Endocr Regul 2021; 55: 101-111.
7. Głowacka B, Chrzęszyk D, Konopka TP. The prevalence and severity of periodontitis in a Polish cross-sectional gerodontological study. J Stoma 2019; 72: 193-201.
8. Marushchak M, Krynytska I, Mazur L, Klishch I, Gabor G, Antonyshyn I. The relationship between experimental alimentary obesity and hard tooth tissues mineralization. Jordan Med J 2017; 51: 25-33.
9. Liu Z, Liu Y, Song Y, Zhang X, Wang S, Wang Z. Systemic oxidative stress biomarkers in chronic periodontitis: a meta-analysis. Dis Markers 2014; 2014: 931083.
10. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 2007; 43: 160-232.
11. Marushchak M, Krynytska I, Petrenko N, Klishch I. The determination of correlation linkages between level of reactive oxygen species, contents of neutrophiles and blood gas composition in experimental acute lung injury. Georgian Med News 2016; 253: 98-103.
12. Marushchak M, Maksiv K, Krynytska I. The specific features of free radical oxidation in patients with chronic obstructive pulmonary disease and arterial hypertension. Pol Merkur Lekarski 2019; 47: 95-98.
13. Zeidán-Chuliá F, Gursoy M, de Oliveira BH, et al. Focussed microarray analysis of apoptosis in periodontitis and its potential pharmacological targeting by carvacrol. Arch Oral Biol 2014; 59: 461-469.
14. Dabiri D, Halubai S, Layher M, et al. The role of apoptotic factors in assessing progression of periodontal disease. Int J Dent Oral Sci 2016; 3: 318-325.
15. Kebschull M, Guarnieri P, Demmer RT, et al. Molecular differences between chronic and aggressive periodontitis. J Dent Res 2013; 92: 1081-1088.
16. Stanisic D, Jovanovic M, George AK, et al. Gut microbiota and the periodontal disease: role of hyperhomocysteinemia. Can J Physiol Pharmacol 2021; 99: 9-17.
17. Tyagi N, Sedoris KC, Steed M, Ovechkin AV, Moshal KS, Tyagi SC. Mechanisms of homocysteine-induced oxidative stress. Am J Physiol Heart Circ Physiol 2005; 289: H2649-2656.
18. Chen Q, Wang Q, Zhu J, Xiao Q, Zhang L. Reactive oxygen species: key regulators in vascular health and diseases. Br J Pharmacol 2018; 175: 1279-1292.
19. Wang XJ, Tian DC, Wang FW, et al. Astaxanthin inhibits homocysteine-induced endothelial cell dysfunction via the regulation of the reactive oxygen species-dependent VEGF-VEGFR2-FAK signaling pathway. Mol Med Rep 2019; 19: 4753-4760.
20. Marushchak M, Lisnianska N, Krynytska I, Chornomydz I. The mechanisms of apoptosis initiation in rats with chronic enterocolitis combined with streptozotocin-induced diabetes. Georgian Med News 2017; 270: 125-130.
21. Stangl GI, Weisse K, Dinger C, Hirche F, Brandsch C, Eder K. Homocysteine thiolactone-induced hyperhomocysteinemia does not alter concentrations of cholesterol and SREBP-2 target gene mRNAS in rats. Exp Biol Med 2007; 232: 81-87.
22. Roth JA, Kaeberle ML. Isolation of neutrophils and eosinophils from the peripheral blood of cattle and comparison of their functional activities. J Immunol Methods 1981; 45: 153-164.
23. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983; 130: 1910-1917.
24. Chechina OYe, Ryazantseva NV, Sazonova YeV, Zhukova NG, Udintseva IN, Novitsky VV. Mechanisms of lymphocyte apoptosis at tick-borne encephalitis. Bulletin of Siberian Medicine 2011; 6: 61-66 [In Russian].
25. Maianski NA, Maianski AN, Kuijpers TW, Roos D. Apoptosis of neutrophils. Acta Haematol 2004; 111: 56-66.
26. Bhardwaj SB, Mehta M, Sood S, Sharma J. Biofilm formation by drug resistant enterococci isolates obtained from chronic periodontitis patients. J Clin Diagn Res 2017; 11: DC01-DC03.
27. Jiang Q, Zhao Y, Shui Y, et al. Interactions between neutrophils and periodontal pathogens in late-onset periodontitis. Front Cell Infect Microbiol 2021; 11: 627328.
28. Gooty JR, Shashirekha A, Guntakala VR, Palaparthi R. Estimation of phagocytic activity of polymorphonuclear leukocytes in chronic and aggressive periodontitis patients with nitroblue tetrazolium test. J Indian Soc Periodontol 2019; 23: 316-321.
29. Shcherba V, Vydoinyk O, Posolenyk L, Korda M. The influence of thyroid hormones on mitochondrial mechanisms of blood neutrophils' apoptosis in case of experimental periodontitis. Arch Balk Med Union 2019; 54: 64-71.
30. Krynytska I, Marushchak M, Svan O, Akimova V, Mazur L, Habor H. The indices of endogenous intoxication in rats with carrageenan solution consumption. Georgian Med News 2018; 279: 196-200.
31. Vitkov L, Hartl D, Minnich B, Hannig M. Janus-faced neutrophil extracellular traps in periodontitis. Front Immunol 2017; 8: 1404.
32. Matthews JB, Wright HJ, Roberts A, Cooper PR, Chapple IL. Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. Clin Exp Immunol 2007; 147: 255-264.

33. Gölz L, Memmert S, Rath-Deschner B, et al. LPS from P. gingivalis and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis. Mediators Inflamm 2014; 2014: 986264.
34. Oktay S, Chukkapalli SS, Rivera-Kweh MF, Velsko IM, Holliday LS, Kesavalu L. Periodontitis in rats induces systemic oxidative stress that is controlled by bone-targeted antiresorptives. J Periodontol 2015; 86: 137-145.
35. Parakhonsky AP, Shmalko NM, Tsyganok SS. The role of leukocyte apoptosis in the immunopathogenesis of periodontitis. Modern Problems of Science and Education 2006; 2: 74-75 [In Russian].
36. Sarkisov AK, Polunina EA, Sarkisov KA. Analysis of the annexin V apoptosis marker level and dental status in patients with chronic generalized perodontitis and bronchoectatic disease. Kuban Scientific Medical Bulletin 2019; 26: 85-92 [In Russian].
37. Lehotsky J, Petras M, Kovalska M, Tothova B, Drgova A, Kaplan P. Mechanisms involved in the ischemic tolerance in brain: effect of the homocysteine. Cell Mol Neurobiol 2015; 35: 7-15.
38. Huang RF, Huang SM, Lin BS, Wei JS, Liu TZ. Homocysteine thiolactone induces apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells. Life Sci 2001; 68: 2799-2811.
39. Kruman II, Culmsee C, Chan SL, et al. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 2000; 20: 6920-6926.
40. Bao XM, Wu CF, Lu GP. Atorvastatin inhibits homocysteine-induced dysfunction and apoptosis in endothelial progenitor cells. Acta Pharmacol Sin 2010; 31: 476-484.
41. Alam MM, Mohammad AA, Shuaib U, et al. Homocysteine reduces endothelial progenitor cells in stroke patients through apoptosis. J Cereb Blood Flow Metab 2009; 29: 157-165.
42. Jang Y, Kim J, Ko JW, Kwon YH. Homocysteine induces PUMA-mediated mitochondrial apoptosis in SH-SY5Y cells. Amino Acids 2016; 48: 2559-2569.
43. Obolenskaya MYu, Rodriges RR, Martsenyuk OP. Folate-related processes in human placenta: gene expression, aminothiols, proliferation and apoptosis. Ukr Biochem J 2011; 83: 5-17.
44. Kim WK, Pae YS. Involvement of N-methyl-d-aspartate receptor and free radical in homocysteine-mediated toxicity on rat cerebellar granule cells in culture. Neurosci Lett 1996; 216: 117-120.
45. Fang M, Jin A, Zhao Y, Liu X. Homocysteine induces glyceraldehyde-3-phosphate dehydrogenase acetylation and apoptosis in the neuroblastoma cell line Neuro2a. Braz J Med Biol Res 2016; 49: e4543.
46. Chen J, Zhuang T, Chen J, et al. Homocysteine induces melanocytes apoptosis via PERK-eIF2α-CHOP pathway 2 in vitiligo. Clin Sci (Lond) 2020; 134: 1127-1141.
47. Zhang Z, Wei C, Zhou Y, et al. Homocysteine induces apoptosis of human umbilical vein endothelial cells via mitochondrial dysfunction and endoplasmic reticulum stress. Oxid Med Cell Longev 2017; 2017: 5736506.