Implementation of programmed cell death in circulating neutrophils and its special characteristics in experimentally induced hyperhomocysteinemia in a setting of thyroid dysfunction

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Cardiovascular (CV) disease continues to be the main cause of morbidity and mortality in worldwide. Hyperhomocysteinemia (HHCy) is a novel metabolic risk factor of vascular damage. In addition to that, there is evidence that HHCy management with folic acid and vitamin B supplements prevents atherosclerosis and its sequelae. Oxidative stress is one of the mechanisms behind the cardiotoxic effects of high homocysteine levels. On the one hand, HHCy facilitates endothelial dysfunction, probably as a result of impaired synthesis and/or inactivation of nitrogen (II) oxide (NO). On the other hand, oxidation of homocysteine is accompanied by formation of reactive oxygen species (ROS), which induce lipid peroxidation in cell membranes and in low density lipoproteins, mitochondrial membrane, secretion of cytochrome C and activation of caspase-3, culminating in apoptosis. Thyroid hormones are known to have a profound effect on CV functions. Hyperthyroidism causes heart rate, myocardial contractility and ejection fraction to increase; this may result in systolic hypertension, systolic heart murmurs, increased left ventricular weight and development of angina and atrial fibrillation with a risk for stroke.

***The aim** of our work was to investigate into the special aspects that characterize implementation of programmed cell death in circulating neutrophils of HHCy rats either without comorbidities or with hyper- or hypothyroidism.*

***Material and methods.** Prolonged hyperthyroidism and hypothyroidism were modeled in experimental rats by dosing the animals with L-thyroxine and thiamazole, respectively, for 21 days, and prolonged with HHCy administered with excessive exogenous HCy, for 21 days. Prolonged HHCy rats with hyper- or hypothyroidism were observed.*

***Results.** We have found the count of circulating neutrophils with increased ROS production and reduced transmembrane mitochondrial potential to be significantly increased in rats with HHCy compared to control animals, which suggests prooxidant properties of HCy and its ability to cause mitochondrial dysfunction. The intensity of ROS production by circulating neutrophils in hyperthyroid animals with HHCy was not significantly different from that in hyperthyroid rats without HHCy. In hypothyroid rats with HHCy, ROS production by circulating neutrophils was significantly higher compared to the control group. HCys increased ROS generation in kidney mitochondria while strongly decreasing it in liver, heart and brain mitochondria showing that the changes are tissue-specific. We have found the count of circulating neutrophils with signs of apoptosis to be increased in rats with HHCy compared to the control group.*

***Conclusions.** Experimentally induced HHCy is accompanied by hyperproduction of reactive oxygen species and by impaired integrity of external mitochondrial membrane, which results in initiation of apoptotic cell death. The deficiency of thyroid hormones enhances initiation of programmed cell death.*

Key words: *hyperthyroidism, hypothyroidism, reactive oxygen species, hyperhomocysteinemia*

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Realizacja programowanej śmierci komórek w krążących neutrofilach i jej szczególne cechy w eksperymentalnie wywołanej hiperhomocysteinemii w warunkach dysfunkcji tarczycy

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Choroby sercowo-naczyniowe (CV) nadal są główną przyczyną zachorowalności i umieralności na całym świecie. Hiperhomocysteinemia (HHCy) jest nowym metabolicznym czynnikiem ryzyka uszkodzenia naczyń. Ponadto istnieją dowody na to, że kontrolowanie HHCy podawaniem kwasu foliowego i suplementów witaminy B zapobiega miażdżycy i jej następstwom. Stres oksydacyjny jest jednym z mechanizmów kardiotoksycznych skutków wysokiego stężenia homocysteiny. Z jednej strony HHCy ułatwia dysfunkcję śródbłonka, prawdopodobnie w wyniku upośledzonej syntezy i/lub inaktywacji tlenku azotu (II) (NO). Z drugiej strony utlenianiu homocysteiny towarzyszy tworzenie reaktywnych form tlenu (RFT), które indukują peroksydację lipidów w błonach komórkowych oraz w lipoproteinach o małej gęstości, w błonie mitochondrialnej, wydzielaniu cytochromu C i aktywacji kaspazy-3, której kulminacją jest apoptoza. Wiadomo, że hormony tarczycy mają głęboki wpływ na funkcje układu krążenia. Nadczynność tarczycy powoduje zwiększenie częstości czynności serca, kurczliwości mięśnia sercowego i frakcji wyrzutowej; może to spowodować nadciśnienie skurczowe, szmery skurczowe serca, zwiększenie masy lewej komory oraz rozwój dławicy piersiowej i migotania przedsionków z ryzykiem udaru.

***Celem** naszej pracy było zbadanie szczególnych aspektów charakteryzujących wdrażanie programowanej śmierci komórkowej krążących neutrofilów u szczurów z HHCy bez chorób współistniejących lub z nadczynnością lub niedoczynnością tarczycy.*

***Materiał i metody**. Długotrwałą nadczynność tarczycy i niedoczynność tarczycy modelowano na szczurach doświadczalnych przez podawanie zwierzętom odpowiednio L-tyroksyny i tiamazolu przez 21 dni oraz z przedłużonym modelem HHCy wywołanym nadmiernym podawaniem egzogennej HCy przez 21 dni. Obserwacją objęto szczury z przedłużoną HHCy z nadczynnością lub niedoczynnością tarczycy.*

***Wyniki.** Stwierdzono, że liczba krążących neutrofilów ze zwiększonym wytwatrzaniem RFT i zmniejszonym transbłonowym potencjałem mitochondrialnym jest znacząco zwiększona u szczurów z HHCy w porównaniu ze zwierzętami kontrolnymi, co sugeruje prooksydacyjne właściwości HCy i jego zdolność do wywoływania dysfunkcji mitochondriów. Intensywność wytwarzania RFT przez krążące neutrofile u zwierząt z nadczynnością tarczycy z HHCy nie różniła się istotnie od tego u szczurów z nadczynnością tarczycy bez HHCy. U szczurów z niedoczynnością tarczycy z HHCy produkcja RFT przez krążące neutrofile była znacznie wyższa w porównaniu z grupą kontrolną. HCys zwiększyło wytwarzanie RFT w mitochondriach nerek, jednocześnie silnie zmniejszając je w mitochondriach wątroby, serca i mózgu, co wskazuje, że zmiany są specyficzne dla tkanki. Stwierdzono, że liczba krążących neutrofilów z objawami apoptozy jest zwiększona u szczurów z HHCy w porównaniu z grupą kontrolną.*

***Wnioski**. Eksperymentalnie indukowanemu HHCy towarzyszy nadprodukcja reaktywnych form tlenu oraz upośledzenie integralności zewnętrznej błony mitochondrialnej, co powoduje rozpoczęcie apoptozy. Niedobór hormonów tarczycy nasila inicjację programowanej śmierci komórki.*

Słowa kluczowe*: nadczynność tarczycy, niedoczynność tarczycy, reaktywne formy tlenu, hiperhomocysteinemia*

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Cardiovascular (CV) disease continues to be the main cause of morbidity and mortality in population worldwide [20,27,30]. Up to 15-20% of patients with cardiovascular disease lack traditional risk factors, such as dyslipidemia, tobacco smoking, hypertension and diabetes [21]. This makes primary prevention impossible and calls for identifying new predictors of cardiovascular disease. Hyperhomocysteinemia (HHCy) (i. e. serum homocysteine levels >15 μmol/L) is a novel metabolic risk factor of vascular damage [9,38,45]. When compared to subjects with homocysteine levels at or below 10.5 μmol/L, patients with serum homocysteine levels >15.3 μmol/L were found to have 1.7 times higher overall cardiovascular mortality, with a 3.4-fold greater risk for fatal myocardial infarction and a 4.3-fold greater risk for fatal stroke [35]. In addition to that, there is evidence that HHCy management with folic acid and vitamin B supplements prevents atherosclerosis and its sequelae [16,44].

Oxidative stress is one of the mechanisms behind the cardiotoxic effects of high homocysteine levels [29]. On the one hand, HHCy facilitates endothelial dysfunction, probably as a result of impaired synthesis and/or inactivation of nitrogen (II) oxide (NO). On the other hand, oxidation of homocysteine is accompanied by formation of reactive oxygen species (ROS), which induce lipid peroxidation in cell membranes and in low density lipoproteins. ROS in turn inactivate NO and trigger the cascade of proinflammatory cytokine-based responses [19]. In addition to that, the excess of ROS causes depolarization of mitochondrial membrane, secretion of cytochrome C and activation of caspase-3, culminating in apoptosis [5].

Thyroid hormones are known to have a profound effect on CV functions [43]. Hyperthyroidism causes heart rate, myocardial contractility and ejection fraction to increase; this may result in systolic hypertension, systolic heart murmurs, increased left ventricular weight and development of angina and atrial fibrillation with a risk for stroke [4]. Patients with subclinical hyperthyroidism may also be at a higher risk for atrial fibrillation [13]. On the other hand, a deficiency in thyroid hormones leads to reductions in heart rate and myocardial contractility and to relaxation of the myocardium [43]. At the same time, thyroid hormones affect the metabolism of homocysteine [1,46].

Therefore, the aim of our work was to investigate into the special aspects that characterize implementation of programmed cell death in circulating neutrophils of hyperhomocysteinemic rats either without comorbidities or with hyper- or hypothyroidism.

MATERIALS AND METHODS

The study has used 48 mongrel male rats weighing 150-180 g. All animals were divided into 6 groups. In group 1 (control group), the animals were administered 1% solution of starch gel intragastrically (n=8). Group 2 included rats with hyperhomocysteinemia (HHCy) administered with excessive exogenous homocysteine (HCy) as homocysteine thiolactone (100 mg/kg of body weight, intragastrically in 1% solution of starch gel once a day for 28 days) (n=8) [40]. Group 3 included animals with hyperthyroidism receiving daily intragastric doses of L-thyroxine 200 μg/kg in 1% solution of starch gel for 21 days (n=8). Group 4 included rats with HHCy receiving daily intragastric doses of L-thyroxine 200 μg/kg in 1% solution of starch gel for 21 days (n=8). Group 5 included animals with hypothyroidism receiving daily intragastric doses of thiamazole 10 mg/kg of body weight in 1% solution of starch gel for 21 days (n=8). Group 6 included rats with HHCy receiving daily intragastric doses of thiamazole 10 mg/kg of body weight in 1% solution of starch gel for 21 days (n=8).

All manipulations with experimental animals were performed according to provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [12].

The experiments have used a population of circulating neutrophils obtained by centrifugation with a dual Ficoll-Verografin velocity sedimentation gradient of 1.077 and 1.093 [31].

Analysis of cell samples to determine all test parameters was performed using an Epics XL flow cytometer (by Beckman Coulter, USA). ROS production was assessed using a blocked fluorescence dye, i.e. dichlorodihydrofluorescein diacetate (DCF-DA) (by Sigma Aldrich, USA) [7]. The values of the test parameter were expressed as a percentage (the ratio of neutrophils with elevated intracellular ROS to the total cell count).

Transmembrane mitochondrial potential ($\Delta\Psi m$) was assessed using a MitoScreen assay kit (by BD Pharmigen, USA) [8], with 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl carbocyanine iodide (JC-1), a fluorochrome, as a key reagent. The results were given as percentages (the ratio of neutrophils with reduced transmembrane mitochondrial potential to the total cell count).

Evaluation of apoptosis/necrosis in population of circulating neutrophils was performed using FITC-labeled annexin V from the "ANNEXIN V FITC" assay kit (by Beckman Coulter, USA) [22]. Discriminant analysis by the type of cell death included the following: Quadrant 1: annexin V-negative and PI-positive cells: necrosis; Quadrant 2: PI- and annexin V-FITC-positive neutrophils: late-stage apoptosis or necrosis; Quadrant 3: PI- and annexin V-FITC-negative neutrophils: viable cells; and Quadrant 4: V-FITC-positive and PI-negative neutrophils: early stage of apoptosis.

Study findings were given as percentage (ratio of annexin-positive cells to total neutrophil count or ratio of propidium iodide-positive cells to total neutrophil count).

Statistical processing of digital data was performed by means of such software as Excel (by Microsoft, USA) and STATISTICA 7.0 (by Statsoft, USA) using parametric methods of data assessment. The values of arithmetic mean (M), dispersion of arithmetic mean and error of the mean (m) were calculated. The probability of difference in values between independent quantitative variables was determined using Student's t-test (the differences were considered significant at $đ<0.05$). The relationships between the test parameters were assessed based on correlation analysis using Pearson's correlation coefficient. The authors calculated linear correlation coefficient (r) and its probability (p), which were properly reflected in the tables (correlation matrices). The correlation coefficient was considered significant at $p<0.05$.

RESULTS

The results of our research study demonstrated a 2.2-fold increase in production of ROS by circulating neutrophils of rats with HHCy ($p<0.001$) relative to the control group (tab. 1). In hyperthyroid rats with HHCy, this parameter was 74.8% higher than in the control group ($p<0.001$); however, when compared to the group of HHCy animals without comorbidities, it was 19.1% lower ($p<0.01$). It is worth noting that the intensity of ROS production by circulating neutrophils in hyperthyroid animals with HHCy was not significantly different from that in hyperthyroid rats without HHCy.

In hypothyroid rats with HHCy, ROS production by circulating neutrophils was significantly (2.7 times) higher compared to the control group, which was a 25.2% ($p<0.01$) positive difference from HHCy animals without comorbidities and a 54.7% positive difference ($p<0.001$) from hyperthyroid animals with HHCy. In the meantime, production of ROS by circulating neutrophils in animals with a model of HHCy-free hypothyroidism was 26.8% higher than that in the control group ($p<0.05$).

A similar trend was observed regarding changes in the count of circulating neutrophils with reduced transmembrane mitochondrial potential ($\Delta\Psi m$). The transmembrane potential may characterize both mitochondrial function and the condition of the cell as a whole, since, in addition to their apparent energetic function (previously regarded as the main mitochondrial function), these organelles not only receive and process proapoptotic signals, but also produce such signals on their own [25]. In rats with HHCy, the count of circulating neutrophils with $\Delta\Psi m$

Table 1. The results for production of reactive oxygen species, transmembrane mitochondrial potential and apoptosis/necrosis of circulating neutrophils in hyperhomocysteinemic rats either without comorbidities or in a setting of hyper- or hypothyroidism (M±m, n=8).
Tabela 1. Wyniki dotyczące produkcji reaktywnych form tlenu, transbłonowego potencjału mitochondrialnego i apoptozy/martwicy krążących neutrofili u szczurów z hiperhomocysteinemią bez chorób współistniejących lub w sytuacji nadczynności lub niedoczynności tarczycy (M ± m, n = 8).

Parameter	Group of animals					
	Intact	HHCy	Hyperthyroidism	HHCy + Hyperthyroidism	Hypothyroidism	HHCy + Hypothyroidism
Suspension of circulating neutrophils						
Count of cells with elevated ROS production, %	18.40±1.40	39.76±2.05 p_1<0.001	31.75±1.02 p_1<0.001	32.16±0.87 p_1<0.001 p_2<0.01	23.34±1.11 p_1<0.05	49.76±1.57 p_1<0.001 p_3<0.01 p_4<0.001
Count of cells with reduced ΔΨm, %	1.38±0.10	2.73±0.14 p_1<0.001	2.40±0.12 p_1<0.001	2.50±0.14 p_1<0.001 p_2>0.05	2.0±0.08 p_1<0.002	3.20±0.13 p_1<0.001 p_3<0.05 p_4<0.01
Count of ANV^+ cells, %	2.08±0.16	3.11±0.18 p_1<0.01	2.80±0.12 p_1<0.01	3.00±0.10 p_1<0.01 p_2>0.05	2.54±0.09 p_1<0.05	3.95±0.15 p_1<0.001 p_3<0.01 p_4<0.001
Count of PI^+ cells, %	1.45±0.10	1.83±0.08 p_1<0.02	1.74±0.07 p_1<0.05	1.90±0.07 p_1<0.01 p_2>0.05	1.60±0.11 p_1>0.05	2.38±0.09 p_1<0.001 p_3<0.002 p_4<0.01

Note: 1. p_1 – the probability of differences between the control group and the test groups; 2. p_2 – the probability of differences between the hyperhomocysteinemic group and the group with hyperhomocysteinemia in a setting of hyperthyroidism; 3. p_3 – the probability of differences between the hyperhomocysteinemic group and the group with hyperhomocysteinemia in a setting of hypothyroidism; 4. p_4 – the probability of differences between the group with hyperhomocysteinemia in a setting of hyperthyroidism and the group with hyperhomocysteinemia in a setting of hypothyroidism.

was found to be increased 2.0 times (p<0.001) compared to the control group. In hyperthyroid rats with HHCy, this parameter was increased 81.2% compared to the control group (p<0.001); that said, there were no significant differences from HHCy animals without comorbidities. In hypothyroid rats with HHCy, the count of circulating neutrophils with reduced ΔΨm was significantly (2.3 times) increased compared to the control group, exceeding the corresponding values by 17.2% (p<0.05) relative to HHCy animals without comorbidities and by 28.0% (p<0.01) relative to hyperthyroid animals with HHCy. In the meantime, in animals with a model of HHCy-free hypothyroidism the count of circulating neutrophils with reduced ΔΨm was 44.9% higher than that in the control group (p<0.001).

In sum, the data obtained in the study suggest hyperproduction of ROS and impaired integrity of external mitochondrial membrane resulting in release of cytochrome C and other proapoptotic proteins from the intermembrane space to the cytosol and the launch of apoptotic cell death. In rats with HHCy, the count of circulating neutrophils with signs of apoptosis was increased by 49.5% (p<0.01) compared to the control group. In hyperthyroid rats with HHCy, this parameter was increased 44.2% compared to the control group (p<0.01); at the same time, there were no significant differences from HHCy animals without comorbidities. In hypothyroid rats with HHCy, the count of ANV^+ circulating neutrophils was significantly increased compared to the control group (by 89.9%); this exceeded the corresponding values by 27.0% (p<0.01) compared to the HHCy animals without comorbidities and by 31.7% (p<0.001) compared to the hyperthyroid animals with HHCy. In the meantime, the count of circulating neutrophils with signs of apoptosis in animals with a model of HHCy-free hypothyroidism was 22.1% higher than that in the control group (p<0.05).

As we assessed the counts of PI^+ circulating neutrophils (which reflect the intensity of necrotic processes in HHCy rats), we found them to be significantly higher (by 26.2%) compared to the control group. In hyperthyroid rats with HHCy, this parameter was increased 31.0% compared to the control group (p<0.01); that said, there were no significant differences from HHCy animals without comorbidities. In hypothyroid rats with HHCy, the count of PI^+ circulating neutrophils was significantly increased compared to the control group (by 64.1%), exceeding the corresponding values by 30.1% (p<0.002) compared to HHCy animals without comorbidities and by 25.3% (p<0.01) compared to hyperthyroid animals with HHCy. That said, the count of circulating neutrophils with signs of necrosis in animals with a model of HHCy-free hypothyroidism was not significantly different from that in controls.

Correlation analysis was performed for the count of circulating neutrophils with increased ROS production and the count of ANV^+ cells. The analysis detected a direct strong correlation in animals with HHCy and in hyperthyroid animals with HHCy (tab. 2); a very strong direct correlation was found in hypothyroid rats with HHCy. That being said, a strong direct correlation was demonstrated between the count of circulating neutrophils with reduced ΔΨm and the count of ANV^+ cells in animals of all test groups.

Table 2. The correlations between circulating neutrophils with increased ROS production, reduced transmembrane mitochondrial potential and signs of apoptosis in hyperhomocysteinemic animals with thyroid dysfunction
Tabela 2. Korelacje między krążącymi neutrofilami ze zwiększoną produkcją RFT, zmniejszonym potencjałem mitochondrialnym przezbłonowym i objawami apoptozy u zwierząt z hiperhomocysteinemią i dysfunkcją tarczycy

Parameter	Count of cells with increased ROS production, %		
Count of ANV^+ cells, %	HHCy	HHCy + Hyperthyroidism	HHCy + Hypothyroidism
	0.74*	0.84*	0.98*
	Count of cells with reduced ΔΨm, %		
	HHCy	HHCy + Hyperthyroidism	HHCy + Hypothyroidism
	0.78*	0.82*	0.81*

Note: * = a significant difference in correlation coefficients, p<0.05

An analysis of correlation between the count of circulating neutrophils with increased ROS production and the count of PI^+ cells has shown a very strong direct correlation in a setting of experimentally induced HHCy and a strong direct correlation in HHCy animals with thyroid dysfunction (tab. 3). Animals with HHCy and hypothyroid animals with HHCy were found to have a strong direct correlation between the count of circulating neutrophils with reduced ΔΨm and the count of PI^+ cells. In the meantime, no significant correlations were found in hy-

perthyroid rats. When analyzing the relationships between the count of ANV⁺ circulating neutrophils and the count of PI⁺ cells, strong direct correlations were found in animals of all test groups.

Table 3. The correlations between circulating neutrophils with increased ROS production, reduced transmembrane mitochondrial potential and signs of apoptosis and necrosis in hyperhomocysteinemic animals with thyroid dysfunction
Tabela 3. Korelacje między krążącymi granulocytami obojętnochłonnymi a zwiększoną produkcją RFT, zmniejszonym potencjałem mitochondrialnym przezbłonowym oraz objawami apoptozy i martwicy u zwierząt z hiperhomocysteinemią i dysfunkcją tarczycy

Parameter	Count of cells with increased ROS production, %		
Count of PI⁺ cells, %	HHCy	HHCy + Hyperthyroidism	HHCy + Hypothyroidism
	0.96*	0.79*	0.85*
	Count of cells with reduced ΔΨm, %		
	HHCy	HHCy + Hyperthyroidism	HHCy + Hypothyroidism
	0.80*	0.62	0.81*
	Count of ANV⁺ cells, %		
	HHCy	HHCy + Hyperthyroidism	HHCy + Hypothyroidism
	0.80*	0.72*	0.87*

Note: * = a significant difference in correlation coefficients, $p<0.05$

DISCUSSION

We have found the count of circulating neutrophils with increased ROS production and reduced transmembrane mitochondrial potential to be significantly increased in rats with HHCy compared to control animals, which suggests prooxidant properties of homocysteine and its ability to cause mitochondrial dysfunction. According to *Medvedev D.V. et al*. [28], the mechanisms behind the toxicity of homocysteine can be divided into primary and secondary mechanisms. The primary mechanisms are likely attributable to the following three types of reactions: 1) reduction of oxygen to the superoxide anion radical in the presence of ions of variable valence metals; 2) NO binding due to nitrosylation of homocysteine; and 3) homocysteinylation, i. e. covalent bonding of homocysteine to sulfhydryl groups of cysteine residues or homocysteine thiolactone to amino groups of lysine residues in protein chains. The secondary mechanisms may include decreasing the effect of NO, increased activity of matrix metalloproteinases and oxidative stress.

As reported by *Signorello M.G. et al.,* homocysteine (HCys) promotes oxidant injury through auto-oxidation, formation of homocysteine mixed disulfides, interaction of homocysteine thiolactones and protein homocysteinylation [39]. There are data that direct incubation in the presence of HCys increases mean ROS levels in endothelial [48] or arterial smooth muscle cells [18]. *Gomez J. et al.* indicate that HCys has capacity for directly modulating the rate of mitochondrial ROS production. However, HCys only increased ROS generation in kidney mitochondria while strongly decreasing it in liver, heart and brain mitochondria showing that the changes are tissue-specific [14]. There is evidence that homocysteine may have effects on the activity of mitochondrial oxidoreductases such as succinate dehydrogenase, thereby altering ROS production by the respiratory chain [39]. However, other studies suggest that HCys-induced increases in ROS levels in endothelial cells are due to induction of NADPH oxidase or decreased thioredoxin expression [41] and therefore are not necessarily due to modifications in mitochondrial ROS production. *Alvarez-Maqueda M. et al*. also estimate that Hcy promotes the formation of ROS primarily by a biochemical mechanism involving endothelial nitric oxide synthase (eNOS) and NADPH oxidase (Nox) activation [3].

Excessive generation of ROS may result in cytotoxic oxidative stress, acting as an upstream factor for mitochondrial membrane depolarization, induces mitochondria to release cytochrome *C* and caspases, resulting in eventual cellular apoptosis [24,26]. We have found the count of circulating neutrophils with signs of apoptosis to be increased by 49.5% in rats with HHCy compared to the control group. Our results are consistent with those reported by other authors, who have demonstrated induction of programmed death in different cell types in a setting of hyperhomocysteinemia. Thus, *Bao X.M. et al*. showed homocysteine-induced apoptosis in endothelial progenitor cells, which may be related to its prooxidative effects as well as an upregulation of p38MAPK protein expression and caspase-3 activity [5]. *Alam M.M. et al*. also demonstrated that Hcy-mediated endothelial cells apoptosis is associated with caspase-8, cytochrome-*c* release, and caspase-3 activation [2]. It has been reported that Hcy induces mitochondrial apoptosis in SH-SY5Y cells [17] and in primary cultures of cyto- and syncytiotrophoblastic cells [32].

When assessing the impact of thyroid status on ROS production, mitochondrial dysfunction and apoptosis of circulating neutrophils, we have found thyroid hormone deficiency to enhance ROS production and initiation of programmed cell death, as supported by a significant numerical predominance of circulating neutrophils with signs of apoptosis in hypothyroid rats as compared to hyperhomocysteinemic animals without comorbidities and compared to hyperhomocysteinemic animals with hyperthyroidism. It appears likely that thyroid hypofunction, on the one hand, enhances oxidative stress due to increased ROS production and low availability of antioxidants, and, on the other hand, increases the levels of homocysteine, the latter possessing pronounced prooxidant properties.

The data concerning association of oxidative stress with hypothyroidism are ambiguous, due to development of a hypometabolic state in cases of thyroid hormone deficiency [23,36,37]. In a group of patients with primary hypothyroidism, *Baskol et al.* [6] found high plasma levels of malondialdehyde (MDA) and NO, and SOD levels, which were not significantly different from those of controls. Elevated MDA levels were also demonstrated in subclinical hypothyroidism [41]. *Erdamar H. et al.* [11] performed an assessment of prooxidant/antioxidant system in 20 patients with hypothyroidism secondary to autoimmune thyroiditis and found an increase in the levels of MDA, nitrite and an increased activity of myeloperoxidase. The authors also concluded on the development of oxidative stress in patients with hypothyroidism, which was moreover more pronounced than that in the group of patients with hyperthyroidism. At the same time, another study conducted on patients affected by subclinical hypothyroidism secondary to *Hashimoto's* thyroiditis did not show any difference in endogenous MDA levels between hypothyroid patients and controls [34].

Excess TSH is also known to directly produce oxidative stress [10].

Increases in plasma homocysteine levels have been reported in overt hypothyroidism and, in some studies, with subclinical hypothyroidism [15, 47]. The possible mechanism responsible for increased HCys level in hypothyroidism also remains a matter of recent debate. Firstly, the observed hyperhomocysteinemia may reflect impaired renal HCys clearance. Hypothyroidism probably reduces glomerular filtration rate leading to increased HCys levels. Secondly, impaired liver metabolism of HCys linked with hypothyroidism may contribute to hyperhomocysteinemia. Decreased activity of both enzymes, methionine synthase and methylenetetrahydrofolate reductase were established in thyroidectomized rats and may also explain the elevated level of HCys in hypothyroidism [33].

CONCLUSIONS

Experimentally induced hyperhomocysteinemia is accompanied by hyperproduction of reactive oxygen species and by

impaired integrity of external mitochondrial membrane, which results in initiation of apoptotic cell death. The correlation analysis performed in this study suggests a significant relationship between parameters of apoptosis, transmembrane mitochondrial potential and production of reactive oxygen species. This indicates a mitochondrial pathway for initiation of apoptosis in both isolated hyperhomocysteinemia and hyperhomocysteinemia combined with thyroid dysfunction. The deficiency of thyroid hormones enhances initiation of programmed cell death, as supported by a significant numerical predominance of circulating neutrophils with signs of apoptosis in hypothyroid rats, a 27.0% ($p<0.01$) positive difference from hyperhomocysteinemic animals without comorbidities and a 31.7% ($p<0.001$) positive difference from hyperhomocysteinemic animals with hyperthyroidism.

REFERENCES

1. Abu-Hassan Diala W, Abdullah N Alhouri, Nadera A, et al.: MTHFR gene polymorphisms in hypothyroidism and hyperthyroidism among Jordanian females. Arch Endocrinol Metab, 2019; 63(3):280-287.
2. 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.
3. Alvarez-Maqueda M, El Bekay R, Monteseirin J, et al.: Homocysteine enhances superoxide anion release and NADPH oxidase assembly by human neutrophils. Effects on MAPK activation and neutrophil migration. Atherosclerosis. 2004;1(72): 229-238.
4. An JH, Song KH, Kim DL, et al.: Effects of thyroid hormone withdrawal on metabolic and cardiovascular parameters during radioactive iodine therapy in differentiated thyroid cancer. J Inter Med Res. 2017;45(1):38-50.
5. Bao XM, Wu CF, Lu GP.: Atorvastatin inhibits homocysteine-induced dysfunction and apoptosis in endothelial progenitor cells. Acta Pharmacol Sin. 2010;31(4):476-484.
6. Baskol G, Atmaca H, Tanriverdi F, et al.: Oxidative stress and enzymatic antioxidant status in patients with hypothyroidism before and after treatment. Exp Clin Endocrinol Diabetes, 2007; 115(8):522-526.
7. Bass DA, Parce JW, Dechatelet LR, et al.: Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol, 1983;130: 1910-1917.
8. Chechyna OE, Ryazantseva NV, Sazonova EV, et al.: Mekhanizmy apoptoza limfocytov pri kleschevom entsefalite [Mechanisms of lymphocytic apoptosis in tick-borne encephalitis]. Byulleten' Sibirskoi Meditsyny – Bull Siberian Med. 2011;6: 61-66 [in Russian].
9. Chrysant SG, Chrysant GS.: The current status of homocysteine as a risk factor for cardiovascular disease: a mini review, Expert Rev Cardiovasc Ther. 2018;16:8, 559-565.
10. Dardano A, Ghiadoni L, Plantinga Y, et al.: Recombinant human thyrotropin reduces endothelium-dependent vasodilation in patients monitored for differentiated thyroid carcinoma. J Clin Endocrinol Metab. 2006;91(10):4175-4178.
11. Erdamar H, Demirci H, Yaman H, et al.: The effect of hypothyroidism, hyperthyroidism, and their treatment on parameters of oxidative stress and antioxidant status. Clin Chem Lab Med. 2008;46(7):1004-1010.
12. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Council of Europe. Strasbourg, 1986;123:52.
13. Franklyn JA, Boelaert K Thyrotoxicosis. Lancet, 2012;379:1155–1166.
14. Gomez J, Sanchez-Roman I, Gomez A.: Methionine and homocysteine modulate the rate of ROS generation of isolated mitochondria in vitro. J Bioenerg Biomembr, (2011); 43:377-386.
15. Gunduz M, Gunduz E, Kircelli F, et al. Role of surrogate markers of atherosclerosis in clinical and subclinical thyroidism. Int J Endocrinol. 2012; 2012:109797.
16. Huo Y, Li J, Qin X, et al. : Efficacy of folic acid therapy in primary prevention of stroke among adults with hypertension in China. The CSPPT randomized trial. JAMA. 2015;313:1325-1335.
17. Jang Y, Kim J, Ko JW, et al.: Homocysteine induces PUMA-mediated mitochondrial apoptosis in SH-SY5Y cells. Amino Acids, 2016;48:2559-2569.
18. Ke XD, Foucault-Bertaud A, Genovesio C, et al.: Homocysteine modulates the proteolytic potential of human arterial smooth muscle cells through a reactive oxygen species dependant mechanism. Mol Cell Biochem. 2010;335:203-210.
19. Krynytska I, Marushchak M The indices of nitrogen (II) oxide system in case of experimental hepatopulmonary syndrome. The Ukrainian Biochemical Journal. 2018;90(5):91-97.
20. Krynytska I, Marushchak M, Zaets T, et al.: Investigation of bone mineralization in patients with coronary heart disease complicated by chronic heart failure, stage II-A. Georgian Med News. 2017;(267):43-48.
21. Kumar A, Khan SA, Parvez A, et al.: The prevalence of hyperhomocysteinemia and its correlation with conventional risk factors in young patients with myocardial infarction in a tertiary care centre of India. Biomed Res. 2011;22(2).
22. Maianski NA, Maianski AN, Kuijpers TW, et al.: Apoptosis of neutrophils. Acta Haematol, 2004; 111(1-2):56-66.
23. Mancini A, Segni C, Di Raimondo S, et al.: Thyroid hormones, oxidative stress, and inflammation. Mediators Inflamm. 2016;2016:6757154.
24. Marushchak M, Krynytska I, Milevska L, et al.: The changes of activity of effector caspase cascade components in case of alimentary obesity in rats. Bangladesh J Med Sci. 2017; 16(2):252-258.
25. Marushchak M, Lisnyanska N, Krynytska I, et al.: The mechanisms of apoptosis initiation in rats with chronic enterocolitis combined with streptozotocin-induced diabetes. Georgian Med News. 2017;9(270): 121-126.
26. 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; 279:95-98.
27. Marushchak M, Krynytska I, Mikolenko A, et al.: Chronic heart failure causes osteopathy or is osteopathy a factor in development of chronic heart failure? Asian J Pharmaceut Clin Res. 2018;11(1): 111-115.
28. Medvedev DV, Zvyagina VI, Uryas'yev OM, et al.: Metabolicheskiye izmeneniya v mitokhondriyakh lyogkikh pri eksperimental'noy gipergomotsisteyinemii u krys [Metabolic changes in pulmonary mitochondria in experimentally induced hyperhomocysteinemia in rats]. Biomeditsinskaya Khimiya – Biomedical Chemistry, 2017;63(3):248-254 [in Russian].
29. Melnyk AV.: Statevi vidminnosti pokaznykiv pro-antyoksydantnoyi systemy v miokardi schuriv za umov gipergomocysteyinemiyi [Gender-specific differences of pro-oxidant system parameters in rat myocardium in a setting of hyperhomocysteinemia]. Zdobutky klinichnoyi i eksperymentalnoyi medytsyny – Adv Clin Exp Med. 2017; 1:47-52 [in Ukrainian].
30. Nechyporuk V, Korda M, Pentiuk L, et al.: The impact of B vitamins on the functioning of methylation cycle in the liver and the kidneys of hyper- and hypothyroid rats. Pol Merk Lekarski. 2020; 48(283):55-59.
31. Neyko YeM, Gerych PR, Ostrovskyi MM et. al.: Kysen'zalezhni funktsiyi fagotsytiv u khvorykh na khronichne obstruktyvne zakhvoryuvannya legen' [Oxygen-dependent functions of phagocytes in patients with chronic obstructive pulmonary disease]. Zdobutky klinichnoyi i eksperymentalnoyi medytsyny – Adv Clin Exp Med. 2010;1:100-104 [in Ukrainian].
32. Obolens'ka MYu, Rodriguez RR, Martsenyuk OP.: Folatzalezhni protsesy u platsenti lyudyny: ekspresiya geniv, aminotioly, proleferatsiya i apoptoz [Folate-dependent processes in human placenta: gene expression, aminothioles, proliferation and apoptosis]. Ukr. biochim. zhurn. – Ukrainian Biochem J. 2011;83(1):5-17 [in Ukrainian].
33. Owecki M, Dorszewska J, Sawicka-Gutaj N, et al.: Serum homocysteine levels are decreased in levothyroxine-treated women with autoimmune thyroiditis. BMC Endocrine Disord. 2014;35: 202.
34. Öztürk Ü, Vural P, Özderya A, et al.: Oxidative stress parameters in serum and low density lipoproteins of Hashimoto's thyroiditis patients with subclinical and overt hypothyroidism. Int Immunopharmacol. 2012;14(4):349-352.
35. Peng H, Chang-Feng M, Xu J, et al.: Elevated homocysteine levels and risk of cardiovascular and all-cause mortality: a meta-analysis of prospective studies. J Zhejiang Univ Sci B. 2015;16(1):78-86.
36. Remenyakina EI, Pavlyuchenko II, Okhremenko OS, et al.: Sravnitelnyi analiz sostoyaniya pro-/antioksidantnoy zashchity u patsientov s disfunktsiei shchitovidnoy zhelezy razlichnogo geneza [Comparative analysis of the status of pro-/antioxidant defense in patients with thyroid dysfunction of various origins]. Sovremennye problemy nauki i obrazovaniya – Modern Problems of Science and Education, 2014;(2):59-62 [in Russian].
37. Savustyanenko AV.: Terapiya pervichnogo gipotireoza á-lipoevoy kislotoy. Obzor rezultatov issledovaniy [Therapy of primary hypothyroidism with á-lipoic acid. A review of research findings]. Mezhdunarodnyj Endokrinologicheskij Zhurnal – Inter J Endocrinol. 2016;4(76):33-40 [in Russian].
38. Schaffer A, Verdoia M, Cassetti E, et al.: Relationship between homocysteine and coronary artery disease. Results from a large prospective study. Thromb Res. 2014;134:288-293.
39. Signorello MG, Vivianib GL, Armanic U, et al.: Homocysteine, reactive oxygen species and nitric oxide in type 2 diabetes mellitus. Thromb Res. 2007;120(4):607-613.
40. Stangl GI.: Homocysteine thiolactone-induced hyperhomocysteinemia does not alter concentrations of cholesterol and SREBP-2 target gene mRNAS in rats. Exp Biol Med. (Maywood), 2007;232(1):81-87.
41. Torun AN, Kulaksizoglu S, Kulaksizoglu M, et al.: Serum total antioxidant status and lipid peroxidation marker malondialdehyde levels in overt and subclinical hypothyroidism. Clin Endocrinol. 2009;70(3): 469-474.
42. Tyagi N, Sedoris KC, Steed M, et al.: Mechanisms of homocysteine-induced oxidative stress. Am J Physiol Heart Circ Physiol. 2005;289:2649-26565.
43. Vargas-Uricoechea H, Bonelo-Perdomo A, Sierra-Torres CH.: Effects of thyroid hormones on the heart. Clinica e investigacion en arteriosclerosis: Publicacion oficial de la Sociedad Espanola de Arteriosclerosis, 2014;26:296-309.
44. Wang ZM, Zhou B, Nie ZL, et al.: Folate and risk of coronary heart disease: a meta-analysis of prospective studies. Nutr Metab Cardiovasc Dis. 2012;22:890-899.

45. Zhang S, Bai YY, Luo LM, et al. : Association between serum homocysteine and arterial stiffness in the elderly: a community-based study. J Geriatr Cardiol. 2014;11:32-38.
46. Zhang Y, Wang Q, Li Q, et al.: Association between hyperhomocysteinemia and thyroid hormones in euthyroid diabetic subjects. BioMed Res Inter. 2015;2015.
47. Zhou Y, Chen Y, Cao X, et al.: Association between plasma homocysteine status and hypothyroidism: a meta-analysis. Int J Clin Exp Med. 2014;7:4544-4553.
48. Zhu WG, Li S, Lin LQ, et al.: Vascular oxidative stress increases dendritic cell adhesion and transmigration induced by homocysteine, Cell Immunol. 2009;254:110-116.

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