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CHEMICAL PHYSICS OF BIOLOGICAL PROCESSES

Investigation of Changes in the Microviscosity of the Erythrocyte Membranes and Glutathione in the Plasma of Animals with an Experimental Pathology Type Alzheimer's Diseases

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Abstract—Changes in the microviscosity of the lipid and protein components of the membrane, lipid peroxidation parameters in erythrocytes based on the level of malonyldialdehyde (MDA), and redox status of the glutathione system in the plasma are studied on NMR1 mice in the experimental model of an Alzheimer's disease (AD)-like pathology from 2 weeks to 12 months after the operation. It is found that the induction of significant levels of lipid peroxidation measured as an increase in the level of MDA, together with AD-like symptoms, occurs only after the significant depletion of reduced glutathione (GSH), increase in oxidized glutathione (GSSG), and decrease in the thiol/disulfate ratio ([GSH]/[GSSG]) in the plasma of ageing mice.

Keywords: viscosity and structure of the membrane, erythrocytes, reduced and oxidized glutathione, blood plasma, experimental pathology, Alzheimer's disease

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INTRODUCTION

One of the most urgent problems at the present time is investigating Alzheimer's disease (AD)-type dementia. Despite significant efforts to study this pathology and the numerous details that are already known, the pathogenesis of AD remains unknown. In developing approaches to create sporadic animal models of AD, the bisection of olfactory bulbs (bulbectomy) was used to initiate diffusive degeneration in specific brain structures. Bulbectomied (BE) animals show an increase in the level of β-amyloid (βA) peptides during the period of the most pronounced spatial memory dysfunction. Neuronal death is recorded in the brain structures related to memory. The described changes in the animal models are viewed as differential characteristics of the AD-type neurodegenerative process in humans [1, 2].

As is well known, Alzheimer's disease is histologically characterized by the so-called neurodegenerative plaques and neurofibrillary tangles forming in neurons that are composed of hyperphosphorylated intracellular tau-protein. The extracellular accumulation of βA leads to local inflammatory processes [3].

Unlike amyloid plaques, inflammatory βA is correlated with neuronal death and decreased cognitive function in AD. This suggests it plays an important role in the progression of the disease. The accumulation of reactive oxygen intermediates in AD may be caused by mitochondrial dysfunction leading to a respiratory chain deficiency and, consequently, to excessive production of reactive oxygen intermediates and the accumulation of extracellular β-amyloid. The accumulation of βA leads to local inflammation and activation of microglia, which is another source of reactive oxygen [4, 5].

The massive synthesis of free radicals increases in the process of cell ageing when the functionality and integrity of the mitochondrial membrane are compromised, thus making its structure and lipid properties sensitive to lipid peroxidation [6].

The lipid component of membranes plays an important role in transferring, processing, and storing information in the cell. Lipid component status in biological membranes is determined by a number of parameters: the microviscosity of lipids, phospholipid and fatty acid composition, and lipid peroxidation (LPO) processes. LPO may be the defining factor in the lipid bilayer status, since it is related to other characteristics of membrane lipids [7].

While studying structural status of erythrocyte membranes in humans with AD, where the structural characteristics were calculated based on erythrocyte hemolysis and the level of malonyldialdehyde (MDA) as the LPO indicator, as well as lipid bilayer microvis-

cosity, all AD patients demonstrated increased viscosity of both the (lipid and periprotein) membrane areas in erythrocytes [8]. At the first stages of an AD-type pathology development in experimental mice, a decrease in the microviscosity of the membranes extracted from the brain was found [9].

The accumulating evidence confirms that oxidative stress and LPO play an important role in most neurodegenerative age-related diseases. Oxidative stress in AD starts early and anticipates cytopathological manifestations. Due to this, oxidative stress may play a key role in the pathogenesis of the disease.

Indeed, cell death and other dysfunctions in AD are related to oxidative stress and oxidative damage. The brain is especially vulnerable to oxidative stress due to the high oxygen intake, lower levels of antioxidants compared to other organs, and large content of lipids [10].

Several balanced systems defining the antioxidant status of the body function in the cell; they can be considered as regulatory systems. A prominent position among them is occupied by enzymes and natural antioxidants—inhibitors of free-radical oxidation. Other antioxidant mechanisms depend to a large extent on sulfur-containing amino acids (cysteine and methionine) in proteins and non-protein cofactors, especially on cysteine-containing tripeptide—reduced glutathione (GSH) which is abundant in cells [11].

The possible role of thiol redox disbalance as a factor promoting neurodegeneration is discussed. The biological activity of sulfur atom in cysteine, which can be free or incorporated into proteins, is a crucial factor in oxidative damage, excitotoxicity, and neurodegeneration. Restoring the redox balance is important for minimizing neuronal death in neurodegeneration [12].

Glutathione is one of the main components of the antioxidant protection system in mammalian cells. It has numerous antioxidant properties, including direct conjugation with free radicals, enzyme neutralization of free radicals, and regeneration of other lipid-soluble antioxidants, such as C and E vitamins [13]. Glutathione inhibits LPO processes by directly blocking the action of reactive oxygen intermediates [14, 15]. Excessive generation of the latter leads to oxidative stress causing a GSH homeostasis disbalance and decreased activity of glutathione-dependent enzymes [12, 16].

Despite the fact that glutathione is synthesized exclusively in the cytosol, it is found in the intracellular pools: cytoplasm, nucleus, and mitochondrias; it is evenly distributed in the intracellular organelles, to control specific compartments and functions when necessary. Intracellular glutathione mainly exists in its reduced form, except for the endoplasmic reticulum where it mainly exists in its oxidized form (GSSG). Oxidized glutathione is the main source of the oxidizing equivalent that creates the microenvironment necessary to form disulfide bonds in proteins. Mitochondrial GSH depletion makes cells more vulnerable to oxidative stress, such as the tumor necrosis factor, hypoxia, and β-amyloid, which underlies the pathogenesis of many diseases [17, 18].

Taking the significant evidence confirming high levels of oxidative stress in the development of neurodegenerative diseases into account, it can be said that finding dementia biomarkers in the symptomless period will help us understand its pathogenesis both in the early and late stages. The experimental model of sporadic AD was based on the hypothesis of the role of olfactory dysfunction in the development of this pathology [1].

Our studies were aimed at investigating the structural characteristics of erythrocyte membranes and analyzing the glutathione system in the plasma of mice in the development of AD-type experimental pathology based on bulbectomy, compared to the sham operation (SO) from two weeks to 12 months after the operation and control mice before the operation.

MATERIALS AND METHODS

The studies were conducted on NMRI female mice [19] before the operation ($n = 19$) and after bisection of olfactory bulbs ($n = 10$) by aspiration through opening the trepanation (bulbectomy). The animals that were exposed to a SO ($n = 9$) underwent similar manipulation but without bulbectomy.

The blood (0.2 mL) drawn from the ophthalmic vein individually from each animal was placed in a citrate solution (0.4 mL) before and 2 weeks, as well as 1, 2, 3, 6, 8, and 12 months, after the SO or bulbectomy. Blood citrate solution was centrifuged for 10 min at 3000 rpm for erythrocyte sedimentation. The glutathione level was measured in the plasma using the technique we described earlier [20]. Sedimentary erythrocytes were washed with a cooled physiological solution 2–3 times. Samples were analyzed in the form of a 5% erythrocyte suspension in a tris-HCl-buffer. The fluidity of the membranes' lipid bilayer was measured by electron paramagnetic resonance (EPR) with spin probes. Stable nitroxide radicals were used as probes [21] 2,2,6,6-tetramethyl-4-capryloyloxylpiperidine-1-oxyl (probe 1) and 5,6-benzo-2,2,6,6-tetramethyl-1,2,3,4-tetrahydro-γ-carbolin-3-oxyl (probe 2) synthesized at the Semenov Institute of Chemical Physics, Russian Academy of Sciences (RAS).

It was demonstrated that probe 1 was mainly localized in the superficial layer (2–4 Å deep) of the membrane's lipid components, whereas probe 2 was located in the lipids adjacent to proteins (2–4 Å deep), which shows the lipid–protein ratio based on the distribution of probe 1 and 2 in the membrane. For convenience, we will further refer to probe 1 as the *lipid probe* and to probe 2 as the *protein probe*. The probes were

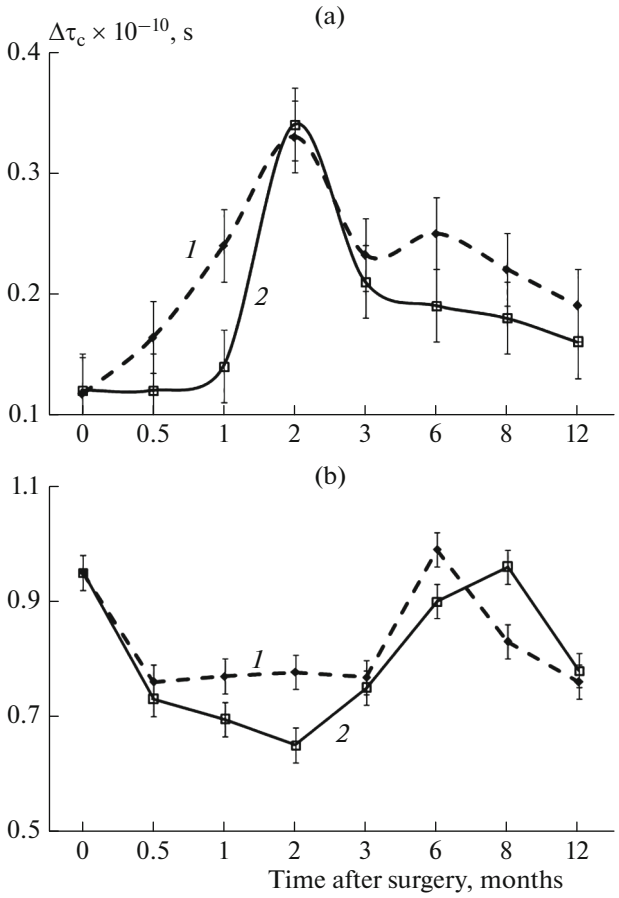

Fig. 1. Changes in the microviscosity of the lipid phase (a) and periprotein areas (b) of erythrocyte membranes in NMR1 mice before (day 0) and after the operation ((*1*) SO animals; (*2*) BE animals) depending on the period.

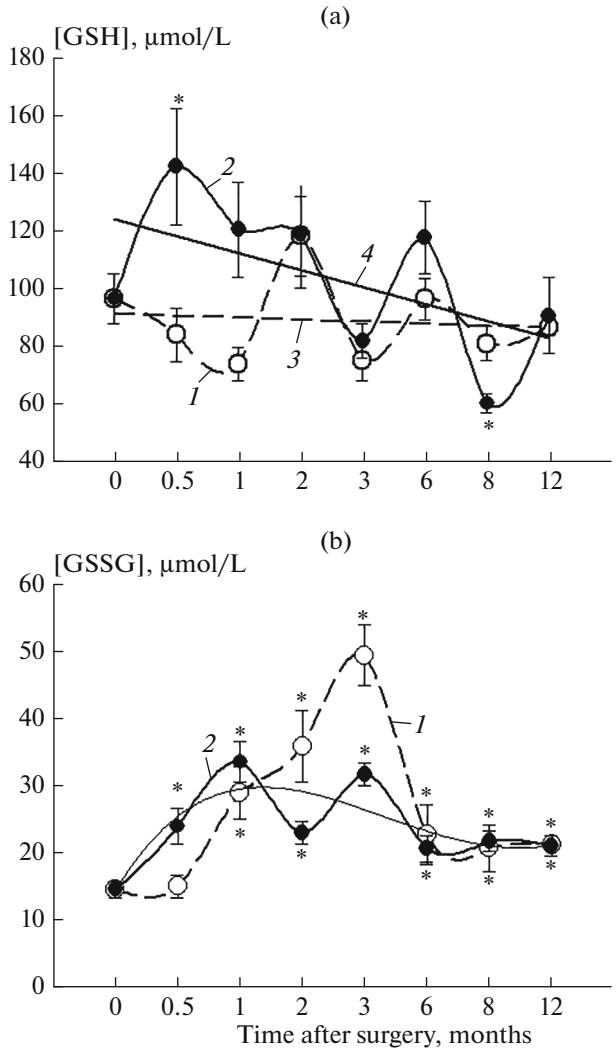

Fig. 2. Changes in the level of GSH (a) and GSSG (b) in the plasma of NMR1 mice before (day 0) and after the operation ((*1*) SO animals; (*2*) BE animals; (*3*) SO animals, correlation coefficient $r = 0.03$, *4*, BE animals, correlation coefficient $r = -0.55$, $n = 8$, $P < 0.05$) depending on the period. Fine solid line in Fig. 2b is the approximation of correlation for BE animals. The differences between groups were considered significant (*) in comparison with controls at $P \le 0.05$.

introduced in samples in the concentration of 10^{-4} M and incubated for 30 to 40 min [22].

Each measurement was repeated 3–5 times. From the obtained EPR spectra, the rotational correlation (τ_c) time was calculated as a quantitative measure of the membranes' component microviscosity using the following formula: $\tau_c = 6.65 \times 10^{-10}\Delta H_+[(I_+/I_-)^{0.5} - 1]$ given in [23]. The EPR spectra were recorded on an ER 200D-SRC radiospectrometer produced by Brucker (Germany).

Statistical analysis was performed using parametric tests in the Microsoft Office Excel, Origin 6.1, and SigmaPlot 10 software at a statistical reliability of 95% ($P < 0.05$).

RESULTS

Blood is the most convenient material in yjr long-term diagnostics of diseases. For this reason, on the one hand we studied changes in the structural characteristics of the erythrocyte membranes and, on the other hand, the changes in the level of the sulfhydryl and disulfide groups in the plasma of BE mice developing a pathological process similar to AD-type dementia, compared to SO and control (prior to operation) animals, at different stages of the disease: from 2 weeks to 1, 3, 6, 8, and 12 months after the operation.

Figure 1 represents changes in the microviscosity of the lipid phase and periprotein areas of the erythrocyte membranes in the SO and BE animals at different times after the operation. The SO animals demonstrate a smooth rise in the microviscosity of the lipid component of the membranes during the first two months after the operation. The τc time characterizing the microviscosity of the membrane components

almost triples in the period from 1 to 2 months. After 2 months the microviscosity of the lipid component of the membranes in SO animals begins to decrease. However, 12 months after the operation, the microviscosity of the lipid phase in the SO animals is higher by a factor of 1.7 times than in the control animals prior to the operation (Fig. 1a).

At the same time, the BE animals show no changes the microviscosity of the membrane lipid phase in the early period after the operation (up to 1 month). The induction period is similar to the control values. After the induction period, the fluidity of the lipid membrane phase in BE animals quickly reaches its peak after two months. However, 2 months after the operation, the microviscosity of the membranes' lipid component in BE animals drops sharply. The τ_c time in BE and SO animals 3 to 12 months after the operation is 1.5 and 1.7 times higher than in the controls, respectively (Fig. 1a).

In the periprotein areas, 2 weeks after the operation, the microviscosity of the membranes in the SO animals decreases from 1.0 to 0.8×10^{-10} s and remains unchanged for 3 months after the operation. Despite the slight rise in the microviscosity in the periprotein areas in the period of 6 to 8 months, after 12 months the microviscosity in the SO animals drops to the level of mice 3 months after the operation. The changes in the microviscosity in the periprotein areas of erythrocyte membranes in BE animals show a similar pattern. However, BE mice 1 to 2 months after the operation demonstrate significantly lower levels of the membrane microviscosity in the periprotein areas compared to SO animals in the same period. By 6–8 months after the operation the microviscosity in the periprotein areas in the SO and BE animals, respectively, reaches the initial level (normal), then toward 12 months after the operation it drops again and becomes approximately half the level found in the controls (Fig. 1b). Thus, the fluidity of both the periprotein and lipid areas of the erythrocyte membranes in BE animals is higher than in SO animals practically at all times after the operation.

The experimental data on the changes in the glutathione status in the plasma of mice before the operation and 2 weeks to 12 months after the operation are presented in Fig. 2. It was found that, at different stages of the development of the disease, the changes in the levels of reduced and oxidized glutathione in the plasma of the control, SO, and experimental animals vary considerably. The levels of GSH in the plasma of BE mice in the early periods after the operation (0.5 months and 1 month) are 1.5 and 1.4 times higher than in the controls, respectively. However, as the disease progresses gradually (2 to 8 months), the glutathione levels drop. Despite the significant increase in the level of reduced glutathione at an early stage (1 month), the level of GSH in the plasma in the period of 0.5 to 12 months shows a negative correlation with the initiation of the neurodegenerative process in BE mice ($r = -0.55$, $n = 8$, $P < 0.05$). As the bulbectomy-induced AD-type neurodegeneration progresses, a significant decrease in the GSH level is observed in the plasma (Fig. 2a).

The SO animals 0.5 and 1 month after the operation show decreased levels of GSH in the plasma; however, by 2 months a slight increase compared to the controls is recorded. In the period of 3 to 12 months after the SO, the level of GSH in the plasma remains unchanged at the same level as in the controls. Despite the oxidative stress caused by the SO, no significant changes in the GSH levels in the plasma of mice depending on the time after the operation are recorded (Fig. 2a).

The SO and BE animals show similar changes in the levels of oxidized glutathione in the plasma practically in the same period. In the period of 2 weeks to 3 months, the animals show increased levels of GSSG in the plasma. The most pronounced increase in the plasma is observed in the SO animals by month 3 compared to the BE animals.

After 6 months, the level of glutathione in the plasma drops and then remains unchanged up to 12 months. Despite the decrease in the level of GSSG in the plasma in the period of 6 to 12 months, it is still significantly higher than prior to the operation. It should be noted that the level of changes in GSSG in the plasma of BE animals in the early and late stages of the neurodegenerative process is half that in SO animals (Fig. 2b).

DISCUSSION

N.V. Bobkova with colleagues from the Institute of Cell Biophysics, RAS (Pushchino) developed the most adequate model among the existing models of sporadic form of AD. To initiate the process of neurodegeneration, bulbectomy is used. The authors note that the period of 2 weeks resembles the early stage of the disease, whereas after 1 month the pathological process reaches its peak according to both the physiological and morphological indicators. The period of 5 months after bulbectomy represents the compensation stage when these indicators go back to normal. Further ageing of the animals is accompanied by the reappearance of AD-like symptoms [1, 2].

The changes observed in AD are attributed to the massive and progressing deterioration of nerve cells caused by various neurological processes. According to the oxidative stress theory, neuronal death results from the free radicals that change the fatty acid composition of the lipids, membrane fluidity, and permeability, thus disrupting the transport functions. One of the byproducts of peroxidation is MDA. Structurally, MDA is a highly-toxic organic substance that takes part in a wide variety of biochemical oxidative reactions. Lipid hydroperoxides are intermediate products

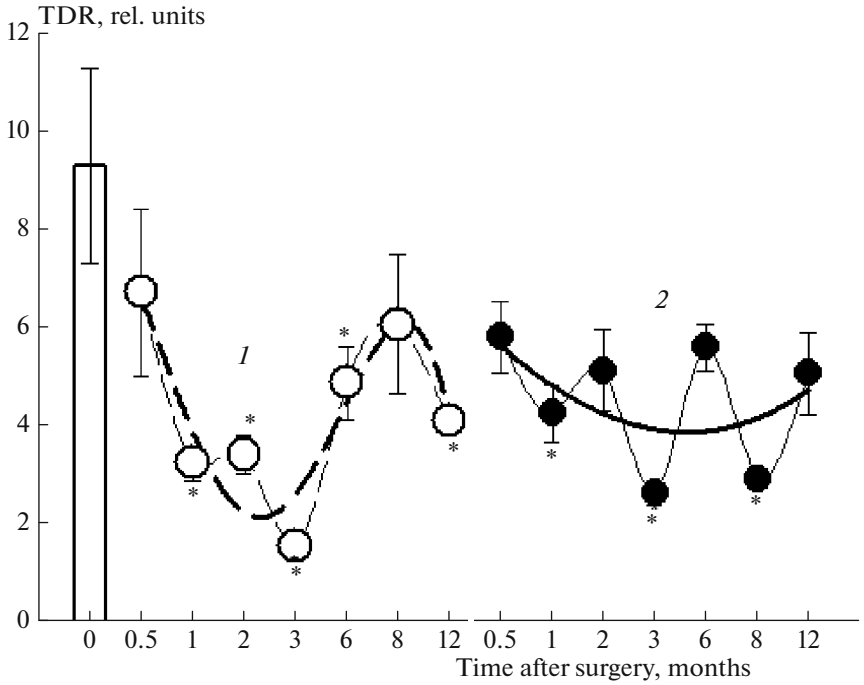

Fig. 3. Changes in the TDR in the plasma of NMR1 mice before (day 0) and after the operation ((*1*) SO animals; (*2*) BE animals) depending on the period. The differences between groups were considered significant (*) in comparison with controls at $P \leq 0.05$.

of lipid peroxidation and can react with unsaturated fatty acids of the membranes, thus affecting their structure [7, 24–26].

In the early stages of the disease, in the BE animals the first change occurs in the structure of the periprotein areas of the membrane related to the functioning of the membrane proteins. In the periprotein areas immediately after the operation, the microviscosity of the membranes in BE animals is practically halved (the fluidity rises) in the period when the disease reaches its peak (1–2–3 months). Following the changes in the periprotein areas, the microviscosity in the BE animals rises and the fluidity of the lipid bilayer decreases 2 months after the operation (Fig. 1). Practically in the same period after the operation (1–2–3 months), the level of oxidized glutathione in the plasma increases in BE animals. However, the level of reduced glutathione rises earlier, 2 weeks after the operation, in the early stage of the disease (Figs. 2a, 2b).

Changes in the thioldisulfate status in the plasma occurs at the same time with the changes in the microviscosity of the periprotein areas of the erythrocyte membranes in BE animals. In the period of 0.5 to 3 months after bulbectomy, the microviscosity of the erythrocyte membranes drops (Fig. 1b) together with the thiol/disulfate ratio (TDR) in the plasma (Fig. 3).

Studying the TDR in the plasma of mice with a bulbectomy-induced AD-type pathology revealed, considerably lower TDR variability is seen in BE mice compared to SO mice. Apart from this, the BE animals show a decrease in the TDR level in the period of 2 weeks to 12 months after the operation, whereas in the SO mice, this decrease occurs in the period of 2 to 3 weeks followed by an increase at the compensation stage (3–6 months); however, the control values are not reached.

On the model of transgenic mice with the double AD-type and wild-type mutation, a quantitative study and comparison of GSH and GSSG and mixed-disulfide proteins (Pr-SSG) were performed both for the brain tissues and blood samples at various stages of the disease (1, 5, and 11 months). Transgenic mice showed a significant drop in the GSH level in the brain tissues and a related decrease in the TDR in all brain regions 5 to 11 months after the operation (immediately prior to the accumulation of βA). In the blood, the level of GSH increased in the period of 1 to 5 months. The rise in the Pr-SSG level in the blood combined with the decrease in the GSSG and Pr-SSG in the brain is attributed to an increased export of GSSG from the brain to the blood in the period of the increased β-amyloid. The overproduction of S-glutathionylated proteins (Pr-SSG) in the blood relates to the following increase in the systemic oxidative stress, which further leads to cell death [16].

The data we obtained using an experimental model of the AD-type pathology in mice was confirmed on the transgenic mice model when the glutathione redox status in the blood was determined.

Table 1. Levels of MDA (μmol/10^6 cells) in the erythrocytes of NMR1 mice before and at different times (0.5, 1, 2, 3, 6, 8, and 12 months) after the operation

Object	Conditions	Controls	MDA level						
			0.5	1	2	3	6	8	12
SO	Before incubation	2.1	2.8	3.7	3.5	4.5	3.7	2.5	3.6
	After incubation	2.8	2.8	4.7	3	5.3	3.3	3.3	4.4
BE	Before incubation	1.9	3.8	4.5	4.5	4.8	5.5	2.2	4.2
	After incubation	2.6	4	9(?)	3.8	3.8	3.3	4.7	4.4

When the levels of MDA in the erythrocytes of BE animals were analyzed, they were seen to rise significantly two weeks after the operation (the early stage of the disease) compared to the controls. Interestingly, at the compensation stage (3–6 months), the level of LPO products decreases; however, in the peak period of the disease, which is 8–12 months after the operation, the MDA levels are twice as high as in the controls.

The SO animals demonstrate a gradual increase in the level of MDA, so that after 12 months it is 1.6 times higher than in the controls. The level of MDA in erythrocytes as an oxidative stress marker rises gradually in BE mice over after the operation. The BE animals demonstrate a similar picture in terms of the MDA levels, albeit, with higher values, which indicates higher LPO levels in the erythrocyte membranes than in the SO animals (Table 1).

The glutathione redox status in the blood serves as an oxidative stress marker. The data obtained by us using BE animals revealed that in the period of the peak development of the disease (8–12 months after the operation), the rise in the MDA levels in the erythrocytes is accompanied by a decrease in the GSH level and an increase in GSSG in the plasma, leading to changes in the TDR (Figs. 2, 3, Table 1).

It is assumed that the decrease in the GSH level in AD may result from the downregulation of GSH homeostasis rather than from the substrate's limitation. GSH cell homeostasis is regulated by the nonallosteric inhibition of the feedback loop: GSH influences the glutamate-cysteine ligase (GCL), which is responsible for the synthesis of the GSH precursor γ-glutamylcysteine (GGC). In the conditions that lead to the downregulation of homeostasis, γ-glutamylcysteine serves as a substrate limiting the critical levels for GSH-syntetase, the main enzyme responsible for the condensation of glycine with GGC to form the final thiole tripeptide—GSH [27].

The clinical trials demonstrated increased levels of oxidative stress markers in patients with mild cognitive impairments similar to dementia patients. These markers include an increase in the level of MDA and a decrease in the level of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (GPX). If the latter is inactivated, oxidative damage to the cell membrane is possible. The target molecules that can be present in various cell structures, such as cell and intracellular membranes, undergo considerable structural and functional changes in this oxidative attack, thus threatening the cell function and resilience [28].

During the reaction catalyzed by GSH peroxidase, the increase in GSSG production may lead to the formation of mixed protein–glutathione disulfates (protein glutathionylation), which is a crucial stage in the redox-regulation mechanism under physiological conditions [29]. The sulfur redox status plays a very important role in the regulation of the cell functions, including signal transduction, the growth of cells, and their survival and death. Reversible and irreversible modifications of cysteine and methionine residues change the protein function or stability, thus disrupting the intracellular sulfhydryl homeostasis. Therefore, the dysfunctions of antioxidant systems and the sulfur redox status is related to the cell's dysfunction, ageing, and various diseases, including AD [30].

The studies undertaken show that dysfunctions of the redox signalization and sulfhydryl homeostasis regulation are likely to enhance the initiation/progression of neurodegeneration. Oxidative stress changes the thioldisulfate status of the key proteins regulating the balance between cell survival and cell death. As for the progression of AD-type diseases, our results support the theory of the early disruptions in GSH homeostasis with its subsequent decrease with advancing age. It is assumed that the lack of GSH synthesis makes the system vulnerable to AD [31].

The most probable explanation of the early events in the development of the experimental pathology is the short paradoxical increase in the level of glutathione. When the redox system becomes unregulated, it creates a disbalance leading to the exceedance of antioxidant protection over the formation of oxidants. It is related to reductive stress, which is characterized by an abnormally high level of reduction components within the biological systems [32].

Reductive stress was also found in animals with an an AD-type experimental pathology when their redox status was determined at a young age, i.e., before the initiation of the disease.

Reductive stress is explained by disruptions in glutathione homeostasis (GSH and TDR increase) and

increased aggregation of proteins in the experiments on mice [33].

Individuals with mild cognitive impairments in the presymptomatic period but with a high risk of AD development (ApoE4 allele carriers) and who are exposed to reductive stress for a long time show an increased expression of glutathione peroxidase catalyzing GSSG reduction [34, 35]. Long-term reductive stress may lead to changes in another remote redox balance, i.e., to oxidative stress, initiating signal disruptions in cells. While oxidative stress is characteristic of the late stage of AD, reductive stress occurs in the early stages of the disease. In the presymptomatic period, reductive stress increases the risk of the further development of AD-type dementia [16].

CONCLUSIONS

In the development of a bulbectomy-induced AD-type pathology, NMRI mice due to oxidative stress demonstrated an increased level of MDA, a byproduct of lipid peroxidation that damages cell structures and macromolecules. It is likely that the main fluidity changes occurred in the periprotein areas of the membrane in BE animals due to these changes. A decrease in the microviscosity of the erythrocyte membranes is observed from the early stage of the disease to its peak manifestation. In the same periods of the manifestation of the disease, an increase in the level of oxidized glutathione in the plasma of animals and a decrease in the TDR is observed. The disruptions in the redox status of the thioldisulfate system in the plasma of BE animals remain as the disease progresses. In fact, the concentrations of GSH and its oxidized form, glutathione disulfate, mainly define the cell's redox status. The fluidity of the periprotein and lipid areas of the erythrocyte membrane in BE animals is higher than in SO animals practically in all periods after the operation. Therefore, changes in the structural state of the membrane and disruption in the regulation of the redox homeostasis of glutathione in animals may be viewed as the result of not only oxidative stress characteristic of the late stages of AD but also reductive (facilitated) stress in the period prior to the disease, which increases the risk of the further development of AD-type dementia.

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