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ORIGINAL ARTICLE

Functioning of the Antioxidant System under Psycho-Emotional Stress

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It was studied intensity of lipid peroxidation and activity of enzymes from antioxidant system in blood plasma and cardiac muscle cells among laboratory rats under 40 days of isolation and violation of diurnal cycle. The received data show that at the background of quantitative changes in NO there also take place changes in the intensity of lipid peroxidation process, indicated by quantitative change in the concentration of Malone dialdehyde and diene conjugates, including Malonedialdehyde and marked with a reduced activity of antioxidant system enzymes, such as catalase and superoxide dismutase activity. Based on the results, we pro-posed that psychological stress is one of the factors contributing to the development of various cardiac diseases.

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It is widely accepted that any kind of stress can cause response reactions in cells of a living organism, namely free radical oxidation, quantitative changes of the intra-cellular calcium, diminution of energy metabolism, and others, eventually ending up with forming a whole list of pathologies, such as pathologies in cardiovascular, digestive and immunological systems, neurodegenerative processes, and mental disorders (Ando *et al*., 2008; Giordano, 2005). For the last few years, special attention has been drawn to studying the influence of these factors on the development of various types of diseases in the cardiovascular system.

The key parameter in alterations of cell metabolism is the activation of lipid per oxidation (LPO). Under normal conditions, this process reaches a fixed level and is necessary for normal functioning of cells. The intensity of LPO depends on appearance of active forms of oxygen and is connected with the degree of functionality of the antioxidant system in a cell (McCord *et al*., 2005; Maekawa *et al*., 2010).

Free radicals, born in the process of functioning of a cell and interacting with molecules of various types, e.g. nitric oxide (NO), initiate the process of

LPO (Cao *et al*., 2000; Levent *et al*., 2006). In its turn NO, as a messenger, takes part in the metabolic processes that determine viability and functional activity of cells. Yet, it is remarkable that under certain conditions it mediates development of pathologic processes as well, e.g. atherosclerosis, heart ischemic and neuro-degenerative diseases, diabetes mellitus, tumors, and others. At the same time, NO diminishes production and secretion of stress hormones, thus contributing to protection of organism from damages caused by stress (Griffiths *et al*., 2002).

An increase in the quantity of NO is noticeable under short- and medium-term stress. On the other hand, reduction in NO quantity is characteristic to exposure to long-term and aggressive stress factors. Such a two-sided effect of NO is caused by its chemical properties. NO easily reacts with oxygen radicals, forming peroxynitrite (Estevez *et al*., 1998; Leza *et al*., 1998). Peroxynitrite stands out with its high reaction potential and causes structural changes in DNA, proteins, and lipids, followed by apoptosis of the cell. This cell destruction process continues until cell's antioxidant system is activated, which aims at protecting the cell from action of molecules of radical nature in excessive amounts. Intensification of pro-oxidant processes that heightens antioxidant abilities of a cell causes another important process, such as oxidative stress. Activation of oxidative stress is the key factor for various diseases of cardiovascular system, such as stenocardia (angina pectoris), chronic cardiac insufficiency, dyslipidemia, arterial hypertension, hyper coagulation, exacerbation of endothelial dysfunction, and vasoconstriction (Griffiths *et al*., 2002).

Antioxidant system of an animal organism is represented by an entire set of endogenous compounds and enzymatic systems (Koshoridze *et al*., 2010; Landmesser *et al*., 2002). Activities of antioxidant enzymatic systems are not constant in cells and undergo changes under certain physiological conditions, especially in case of a long-term and aggressive stress. It has been found that isolation of an individual animal and disruptions of circadian rhythm pertain to this kind of stress (Burjanadze *et al*., 2009; Pinna *et al*., 2003; Koike *et al*., 2009).

Proceeding from what has been mentioned above; the aim of our investigation is to determine the level of activity of LPO process and antioxidant system in blood and cardiac muscle cells of Wistar rats under isolation and disruption of their circadian rhythm.

MATERIALS AND METHODS

Animals and social conditions

The experiment was conducted on 90 adult male Wistar rats (348 ± 5 g) divided into four groups. Rats in Groups 1, 2, and 3 (socially isolated rats, SI rats), 15 in each group, were isolated in individual cages in the dark (dark-to-light ratio, 23.5/0.5 h) during 20, 30, and 40 days, respectively. Control group (Group 4) contained 45 animals kept in a common cage under natural conditions (dark-to-light ratio, 14/10 h). During the experiments the rats were given water and a standard laboratory chow *ad libitum*. The experiment was repeated four times.

Preparation of samples

Blood and plasma taken from the abdominal vein were used in our experiments. Mitochondrial and cytosole fractions were extracted from cardiac muscle cells (Schlegel *et al*., 1988). During the experiment, the rats were put to sleep by means of chloroform and decapitated under cold

temperature to extract the heart. The experiments were conducted in full accordance with the legal and statutory acts applicable in Georgia and the international agreements ratified by the country, such as the Law of Georgia on Health Care and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Activities of antioxidant system

Amount of NO was measured by determination of the product ($NaNO_2$) of reaction between NO and molecular oxygen (O_2) (Pahan *et al*., 2000). Concentration of active products of thiobarbituric acid, including malondialdehyde, was determined in experimental samples by means of thiobarbituric acid test at the wavelength of 532 nm (Michara and Uchiyama, 1978). Diene conjugates of non-saturated fatty acids were determined by spectrophotometry (Skornyakov *et al*., 1988). The method of determining catalase activity was based on the ability of hydrogen peroxide to form a colored complex with salts of molybdenum, and intensity of their coloring was measured at the wavelength of 410 nm. The principle used to determine superoxide dismutase (SOD) proceeds from the ability of the enzyme to compete with tetrazole nitro blue for superoxide anion radicals (Tasset *et al*., 2008).

Determination of hormone level in the blood

We defined the content of Melatonin, Serotonin and Corticosterone in the animal blood by ELISA Kits ("IBL, international", Germany), according to the manufacturer's protocol. Protein concentration was measured with Protein Assay Kit (Sigma, USA), according to the manufacturer's protocol. All the reagents were purchased from Sigma–Aldrich (Sigma–Aldrich Inc., St. Louis, USA) unless otherwise specified.

Data analysis

Significance for tests was set at $P< 0.05$. The data from each biochemical experiment were analyzed separately and treated by analysis of variance (ANOVA). Experiments were repeated four times with triplicate samples for each experiment. When the significant effect was observed by ANOVA, Student's t-test was also used to compare the samples

RESULTS

Definition of Psycho-Emotional State of Animals under Chronic Stress

Prolonged social isolation and violation of the natural diurnal cycle among animals has been considered an important psychological stress-factor (Maekawa *et al*. 2010). Therefore, at the initial stage of the experiments we studied psycho-emotional state of the animals under isolation and violation of diurnal cycle at the example of the change in the intensity of research and phobic reactions. The received results are presented in Table 1 demonstrating that prolonged (40-day) isolation and violation of the natural diurnal cycle significantly affects their physiological properties, namely increasing the number of those indicating growing anxiety and emotional tension in the animals. This is accompanied by a significant reduction in research reactions and locomotors activity. Existence of stress caused by isolation and disruption of the diurnal cycle is also indicated by the data demonstrated by quantitative changes of such hormones in the blood plasma as Corticosterone, serotonin and melatonin (Table 2).

The obtained results show that after 40 days of stress, concentration of Corticosterone decreases by approximately 27% in the blood plasma, while

quantity of serotonin increases by around 85%. The data also indicate that blood plasma of stressed laboratory animals contains a radically increased quantity of melatonin. Namely, on the 40th day of stress its concentration is approximately 3 times higher.

Literary data show that quantitative reduction of Corticosterone is a change characteristic under chronic stress. On the other hand, short and intense stress leads to an increase in its content (Sanchez *et al*. 1998; Malkesman *et al*. 2006; Djordjevic *et al*. 2010). Thus, as we can see from the results, a 40-day isolation and disruption of diurnal cycle results in the development of significant stress among animals.

Quantitative changes in NO and LPO products under stress By means of the study performed at initial stages, we found that isolation and disruption of the circadian rhythm caused non homogeneous changes in concentrations of NO in mitochondrial and cytosolic fractions of blood cells and cardiac muscle cells in Wistar rats (Table 3). There was a noticeable decrease in NO concentration in blood after 30 days of stress. The concentration of NO was reduced by 42% in comparison with the control data on the 40th day of the isolation in blood, which is smaller compared with the decrease in mitochondria of cardiac muscle cells. Meanwhile the concentration of NO in cytosol of cardiac muscle cells was increased by 14% compared with the control. Next, we studied the content of total lipids in blood cells and cardiac muscle cells.

As shown in Table 4, dynamics of the change in total lipids is non-homogeneous. For instance, an increase in the content of lipids by 22% in cardiac muscle cells was detected after 20 days of isolation, but in blood the content of lipids was increased by 65% after 30 days of stress. As is known, NO is connected with a broad range of cellular processes as a signal molecule. It can diminish the generation of ATP by inhibiting oxidative phosphorylation, followed by appearance of superoxide radicals and peroxynitrite in mitochondria. This, in its turn, causes super oxidation of phospholipids in the mitochondrial membrane and contributes to oxidation of membrane proteins and enzymes. Then, we studied quantitative changes of malondialdehyde and diene conjugates, the products of LPO, in blood and cardiac muscle cells of rats exposed to 40 days of stress. The results are presented in Table 5. The 20-day-long stress caused an increase in malondialdehyde and diene conjugates in cardiac muscle cells and blood cells as well, and this increase was enlarged with the stress time prolonged. For instance, on the 30th day of isolation and disruption of circadian rhythm among the animals, malondialdehyde was increased about four times in mitochondria of cardiac muscle cells. Similar situation was also observed for the quantities of diene conjugates, which points to an increase in the intensity of LPO.

Malondialdehyde can interact with protein molecules and nucleic acids, causing the formation of intermolecular bonds, by which malondialdehyde can lead to structural alterations in various receptors, ionic channels, cytoskeleton, proteins, enzymes, and nucleic acids. Moreover, malondialdehyde also can change the activity of antioxidant system in a cell as well as that of the enzymes involved in it. Antioxidant system of the cell develops an effective response to preserve the homeostasis of the cell (Heitzer *et al*., 2001). The antioxidant system is a multicomponent system and involves a broad range of compounds and enzymatic systems, among which SOD and catalase are most remarkable. Bearing this in mind, our next

step was to study the dynamics of the changes in SOD and catalase activity under the corresponding stress condition. As shown in Table 6, at the condition of 20 days of isolation, there was a remarkable increase in the activity of SOD in mitochondria of cardiac muscle cells, and after that the activity of enzyme was inhibited. On the 40th day, the activity of enzyme was diminished by almost 50% in comparison with the control, indicating the growing of superoxide radicals in excessive amounts. The similar change curves on enzyme activity in cytosolic isoform of cardiac muscle cells and blood as well were observed. Changes were also found in catalase activity. In all cases, the decrease in the enzyme activity is detected after 20-day-long stress.

Table 1. Physiological characteristics of animals under the stress caused by isolation and disruption of diurnal rhythm

		Control (0-day stress)	40-day stress
Fear reactions	Defecation	3,6±0.9	5,6±0.3*
	Duration of grooming (sec)	3.7±0.5	17,2±1.4**
	Number of freezing	3,2±0.8	9,8±2.5**
	Duration of freezing (sec)	14,4±2.6	21,4±2.4**
	Vertical stand	11,6±2.4	3,2±0.7**
Research reactions	Duration of position in the centre (sec)	4.0±0.7	2.2±1.1*
	Number of position in the centre	1.9±0.4	0.8±0.1*
	Number of centripetal movements	5.3±1.3	0.6±0.1**
	Number horizontal movements	4.0±1.5	0**

*P≤0.05; **P≤0.001

Table 2. Change in hormones (nmol/L) under the stress caused by isolation and disruption of diurnal rhythm

Hormone	Control	40-day stress
Corticosterone	289.9±10.4	210.0±16.7*
Serotonin	3.9±1.8	7.2±1.9*
Melatonin	15.9±6.3	48.5±16.9**

*P≤0.05; **P≤0.001

Table 3. Dynamics of the quantitative changes of NO concentrations (mM) in blood and cardiac muscle cells of Wistar rats under stress

Sample	Control	20 days of stress	30 days of stress	40 days of stress
Mitochondria (cardiac muscle cells)	0.16±0.20	0.14±0.06	0.11±0.03*	0.09±0.07**
Cytosol (cardiac muscle cells)	0.35±0.02	0.33±0.06	0.39±0.04*	0.40±0.09*
Blood	0.88±0.11	0.91±0.13	0.66±0.01**	0.51±0.08**

*P <0.05, **P<0.001 compared with the control.

Table 4. Dynamics of the changes in the contents of total lipids (mg/ml) in blood and cardiac muscle cells of Wistar rats under stress

Sample	Control	20 days of stress	30 days of stress	40 days of stress
Cardiac muscle cells	2.04±0.33	2.50±0.21*	2.15±0.10*	2.20±0.40*
Blood	3.42±0.32	3.40±0.14	5.64±0.17**	7.12±0.91**

*P <0.05, **P<0.001 compared with the control.

Table 5. Alterations of quantities of the products of lipid peroxidation in blood and cardiac muscle cells of Wistar rats under stress

	Malondialdehyde (nM/mg protein)				Diene conjugates (nM/mg protein)			
Sample	**Control**	**20 days of stress**	**30 days of stress**	**40 days of stress**	**Control**	**20 days of stress**	**30 days of stress**	**40 days of stress**
Mitochondria (cardiac muscle cells)	0.59±0.10	0.88±0.08*	2.33±0.16**	3.30±0.33**	0.98±0.05	1.03±0.01	3.13±0.18**	4.02±0.84**
Cytosol (cardiac muscle cells)	0.98±0.12	1.05±1.03	2.40±0.27**	2.47±0.19**	1.37±0.23	2.53±0.47**	4.04±1.37**	5.89±1.29**
Blood	2.08±0.25	2.98±0.06*	5.01±0.09**	6.61±1.45**	1.18±0.10	2.88±0.20**	2.97±0.42**	5.34±0.57**

*P <0.05, **P<0.001 compared with the control.

Table 6. Changes in activities of antioxidant system enzymes (U/mg protein) in blood and cardiac muscle cells of Wistar rats under stress

Sample	Control	20 days of stress	30 days of stress	40 days of stress
SOD in mitochondria	16.79±1.09	23.30±2.35**	10.99±2.12**	8.42±3.43*
SOD in cytosol	5.41±0.59	8.10±1.02**	4.01±2.22*	2.72±0.26**
Catalase	12.90±3.00	11.30±2.09	10.70±1.60**	4.80±0.80*
Blood **SOD**	16.25±1.34	16.35±1.73	11.78±0.98**	9.08±1.35**
Blood **Catalase**	21.80±1.96	19.06±1.26*	17.42±2.12**	14.67±3.06**

*P <0.05, **P<0.001 compared with the control.

DISCUSSION

It has been stated that durable social isolation causes emotional stress among laboratory animals. The stress, in turn, causes various behavioral disturbances, increased aggressiveness (Kunieda *et al*., 2006; Pinna *et al*., 2003; Kekelidze *et al*., 2001). Accordingly, social isolation can be considered as one of the psychological stress factors. Multiple studies have already certified the existence of a link between social isolation and pathologies of various kinds, including cardiovascular diseases. In general, stress represents one of the factors altering the mechanism of cardiovascular system. Under the influence of stress, an amount of circulating corticosteroids increases in blood, activating glucocorticoid receptors and producing reactive oxygen radicals that in turn serve as the cause of development of various pathologies of the cardiovascular system (Pinna *et al*., 2006).

As it has been already established, one of the

targets of reactive oxygen radicals is activation of LPO. This process begins in cells due to exposure to various kinds of stress factors (emotional, physical, psychological, etc.) (Nulton-Persson and Szweda, 2001; Pinna *et al*., 2003). Under normal conditions, nascence of reactive oxygen radicals are always maintained to a certain degree, as cells are equipped with an antioxidant system that balances the process. Disruption of the balance between appearance of free radicals and activity of antioxidant system becomes the causative factor of oxidative stress. It is regarded that oxidative stress serves as the main molecular mechanism of damage to the heart and vessels, resulting in diseases such as chronic cardiac insufficiency, atherosclerosis, hyperlipidemia, arterial hypertension, cardiac muscle cells, and others. In the past few years, oxidative stress and its prevention has become an object of active research. Molecules of various types take part in oxidative stress including NO, lipid-free radicals ($LOO^{\cdot}$), hydrogen peroxide (H_2O_2), and so on. In permissible concentrations, each of these represents an intermediate product of a cell's life cycle. Apart from this, many of them, e.g. NO, act as a messenger for many intra- and extra-cellular processes. On the other hand, under insufficiency of the antioxidant system local processes can be generalized and result in damaging cellular proteins, DNA and membrane lipids. NO has a special role in this process, particularly in the formation of diseases of the cardiovascular system. As it becomes clear from the obtained results, stress caused by isolation and circadian rhythm was reflected in blood by reduction of NO concentration (Table 3), which, in turn, is closely linked with a dangerous disease such as atherosclerosis. Augmented quantities of active forms of oxygen in blood, the indicator of increased LPO products, malondialdehyde and diene conjugates, under stress (Table 5) will damage endothelial cells and cause decrease in secretion of NO. This is followed by deepening of endothelial dysfunction, which is revealed in vasoconstriction, hypo coagulation, and proliferation of smooth muscle cells. At the same time, free radicals can damage cardiac muscle cells and contribute to the structural modification of their lipid bilayer, in which the latter will have a negative impact on their contractile function. It is remarkable that the increase in the quantities of LPO products is characteristic of cardiovascular diseases in general (Kunieda *et al*., 2006; Sevanian *et al*., 2000).

In case of social isolation of animals, decrease in the activity of antioxidant enzymes SOD and catalase in blood as well as in cardiac muscle cells serves as an index that oxidative stress has been activated. Suppression of contractile abilities of myocardium by active radicals represents an important mechanism in cardiovascular diseases. The interaction of active radicals with membrane lipids gives rise to lipid radicals, causing an increase in the permeability of the cardiac muscle cell membrane. In its turn, this is followed by an increase in the contents of calcium and extent of the degree of contraction of myofibrils. The main result of these processes is the damage to the function of myocardial elasticity and diminution of its contractile function (Weiss and Lamp, 1987). Thus, isolation of the animals and disruption of their circadian rhythm represent a stress factor that causes biochemical transformations in blood and cardiac muscle cells that leads to a broad range of pathologic conditions of the cardiovascular system.

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