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Original Article

Functional state of rat cardiomyocytes and blood antioxidant system under psycho-emotional stress

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We studied the functionality of the antioxidant system in laboratory rat cardiomyocytes and blood under psycho-emotional stress. It was found that 40-day isolation and violation of diurnal cycle among the animals were accompanied by the intensification of lipid peroxidation process and marked with a reduced activity of antioxidant system enzymes, such as catalase and superoxide dismutase activity. The results suggested that psycho-emotional stress was accompanied by oxidative stress, causing a reduction in the intensity of energy metabolism in cardiomyocytes, which was further strengthened by the fact that the activity of the enzymes involved in ATP synthesis in mitochondria was reduced. Based on the results, we proposed that psychological stress is one of the factors contributing to the development of various cardiac diseases.

Keywords cardiomyocytes; lipid peroxidation; stress; nitric oxide; superoxide-dismutase; catalase

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Introduction

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 intracellular 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 [1,2]. 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 peroxidation (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 [3,4].

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 [5,6]. 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 [7].

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 [8,9]. 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, hypercoagulation, exacerbation of endothelial dysfunction, and vasoconstriction [7].

Antioxidant system of an animal organism is represented by an entire set of endogenous compounds and enzymatic systems [10,11]. 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 [12–14].

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 cardiomyocytes 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 cardiomyocytes according to Schlege *et al*. [15]. 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) [16]. 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 [17]. Diene conjugates of nonsaturated fatty acids were determined by spectrophotometry [18]. 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 [19].

Activity of mitochondrial enzymes

For determining the activities of aconitase, fumarase, and aldolase freshly isolated mitochondria were suspended in 0.5 ml of buffer containing 50 mM Tris–HCl (pH 7.4) and 0.6 mM $MnCl_2$ and sonicated for 2 s. Aconitase activity was measured spectrophotometrically by monitoring the formation of *cis*-aconitate from added iso-citrate (20 mM) at 240 nm and 25°C. One unit (U) was defined as the amount of enzyme necessary to produce 1 μmole *cis*-aconitate per minute (ϵ_{240}= 3.6 mM^{-1} cm^{-1}). Fumarase activity was determined by measuring the increase in absorbance at 240 nm at 25°C in the reaction mixture to which 30 mM potassium phosphate (pH 7.4) and 0.1 mM L-malate were added. One unit was defined as the amount of enzyme necessary to produce 1 mM fumarate per minute (ϵ_{240}= 3.6 mM^{-1} cm^{-1}). Hydrazine assay was used to measure aldolase activity using D-fructoso-1,6-diphosphate (Sigma, St Louis, USA) as substrate and hydrazine sulfate (Sigma) as detection reagent for the 3-phosphoglyceraldehyde formed. One unit is described as absorbance (A) change per minute at 25°C, pH 7.5, and 240 nm wavelength (U/mg = ($A_{240test}$ − $A_{240blank}$)/mg enzyme per ml reaction mixture) [20].

In order to determine the activity of succinate dehydrogenase, freshly isolated mitochondria were suspended in 0.5 ml of buffer containing 50 mM Tris–HCl (pH 7.4) and sonicated for 2 s. Mitochondria were incubated with 2% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide in a solution of 0.05 M Tris, 0.5 mM $MgCl_2$, 2.5 mM $CoCl_2$, and 0.25 M disodium succinate (Sigma-Aldrich) at 37°C for 30 min. Mitochondria were solubilized by adding 150 μl of 6.35% dimethyl sulfoxide (prepared in 0.1 N NaOH) and colored formazan product was measured using a microplate reader at a wavelength of 560 nm. One unit was defined as the amount of enzyme necessary to produce 1 μmole formazan per minute (ϵ_{560}= 17 mM^{-1} cm^{-1}) [21].

Activity of creatine kinase was determined by NADH formation following absorbance at 340 nm at 25°C. The assay medium contained 0.1–0.15 mg/ml of the mitochondrial protein, 50 mM Tris−HCl, pH 7.4, 10 mM glucose, 5 mM $MgCl_2$, 2 mM ADP, 1 mM NAD^+, 5 U/ml yeast hexokinase, and 1 U/ml glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*). The reaction started when 5 mM creatine phosphate was added. Change in absorbance over time at 340 nm was measured, and NADH generation rate was determined. One unit was defined as the amount of enzyme necessary to produce 1 μmole of NADH per minute at 25°C [22].

All the reagents were purchased from Sigma-Aldrich 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

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 cardiomyocytes in Wistar rats (**Table 1**). 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 cardiomyocytes. Meanwhile the concentration of NO in cytosol of cardiomyocytes was increased by 14% compared with the control.

Next, we studied the content of total lipids in blood cells and cardiomyocytes. As shown in **Table 2**, dynamics of the change in total lipids is non-homogeneous. For instance, an increase in the content of lipids by 22% in cardiomyocytes 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 phosphorilation, followed by appearance of superoxide radicals and peroxynitrite in mitochondria. This, in its turn, causes superoxidation 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 cardiomyocytes of rats exposed to 40 days of stress. The results are presented in **Table 3**. The 20-day-long stress caused an increase in malondialdehyde and diene conjugates in cardiomyocytes 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 cardiomyocytes. 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 [23]. The antioxidant system is a multicomponent system and involves a broad range of compounds and enzymatic

Table 1 Dynamics of the quantitative changes of NO concentrations (μM) in blood and cardiomyocytes of Wistar rats under stress

	Control	20 days of stress	30 days of stress	40 days of stress
Cardiomyocytes				
Mitochondria	0.16 ± 0.20	0.14 ± 0.06	0.11 ± 0.03*	0.09 ± 0.07**
Cytosol	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**

*$P \leq 0.05$, **$P \leq 0.001$ compared with the control.

Table 2 Dynamics of the changes in the contents of total lipids (mg/ml) in blood and cardiomyocytes of Wistar rats under stress

Sample	Control	20 days of stress	30 days of stress	40 days of stress
Cardiomyocytes	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**

*$P \leq 0.05$, **$P \leq 0.001$ compared with the control.

Table 3 Alterations of quantities of the products of lipid peroxidation in blood and cardiomyocytes of Wistar rats under stress

	Malondialdehyde (nM/mg protein)				Diene conjugates (nM/mg protein)			
	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
Cardiomyocytes								
Mitochondria	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	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**

*$P \leq 0.05$, **$P \leq 0.001$ compared with the control.

Table 4 Changes in activities of antioxidant system enzymes in blood and cardiomyocytes of Wistar rats under stress

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

*$P \leq 0.05$, **$P \leq 0.001$ compared with the control.

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 4**, at the condition of 20 days of isolation, there was a remarkable increase in the activity of SOD in mitochondria of cardiomyocytes, 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 cardiomyocytes 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.

Activity of energy metabolism enzymes under stress

The resulting data showed that isolation and violation of the natural circadian rhythm caused the activation of oxidative processes in the blood and cardiomyocytes among rats, as well as the deterioration in the functionality of the antioxidant system, which in turn might have an impact on the activity of various enzymes, including the mitochondrial enzymes involved in energy metabolism. The studied enzymes included aconitase, an enzyme from the citrate cycle. As previous studies suggest [21,24], the active center of aconitase is represented by an iron-sulfurous cluster, which is the reason why it shows high sensitivity toward active forms of oxygen. It can be possibly used as a specific marker, sensitive to the oxidative stress. As **Fig. 1(A)** shows, on the 40th day of stress, the activity of aconitase in mitochondria of cardiomyocytes was diminished by 70% compared with the control. Unlike aconitase, fumarase, another enzyme of the same cycle, did not show sensitivity to the oxidative substances and its activity insignificantly changes during the period of stress [**Fig. 1(B)**]. Thus, we assume that during the long-lasting social isolation, mitochondrial enzymes are damaged in rat cardiomyocytes, and the cause of this process might presumably lie in the oxidative stress.

The next enzyme to be studied was succinate-dehydrogenase that plays an important role both in functionality of the respiratory chain and in the citric cycle in mitochondria. Deficiency of any kind of this enzyme is believed to be incompatible with life [20,25]. It was found that 40 days of isolation caused the decrease in the activity of this enzyme for 40% [**Fig. 1(C)**], which pointed to the decrease in oxidative phosphorylation taking place in mitochondria, and the decrease in ATP generation and augmentation of oxidative stress. Changes in the activity of enzyme creatine phosphokinase also showed the same process [**Fig. 1(D)**]. It is already ascertained that creatine

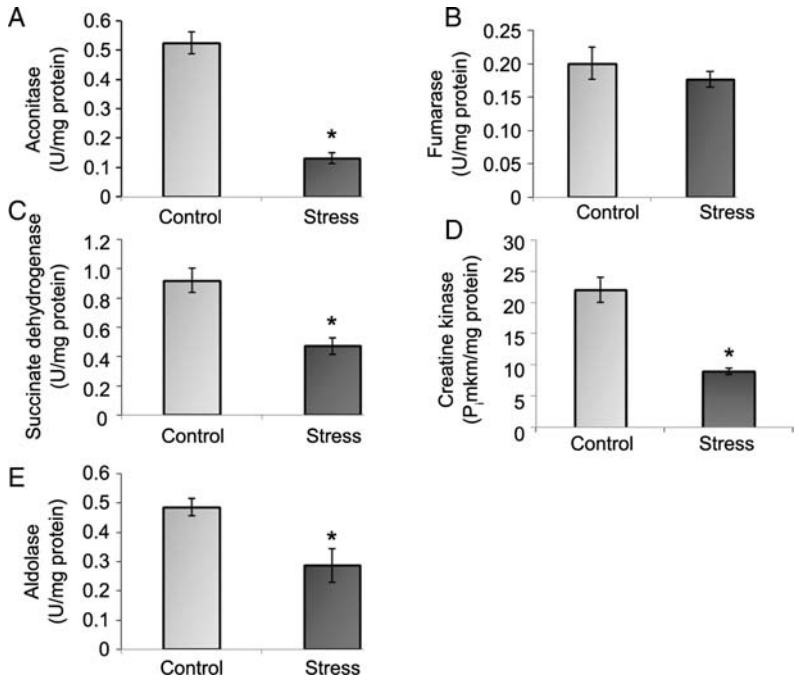

Figure 1 Changes in enzyme activities in cardiomyocytes of Wistar rats under 40 days of stress Comparison of the activities of aconitase (A), fumarase (B), succinate dehydrogenase (C), creatine kinase (D), and aldolase (E) in the mitochondria between control and social isolated (SI) rats was performed using *t*-test. Values of the data are presented by the results of four separate sessions. $*P < 0.05$ compared with the corresponding control group.

phosphokinase is actively involved in the process of balancing ATP quantity by means of the creatine/creatine-phosphokinase/creatine-phosphate system [26].

It is believed that cardiomyocytes stand out with a high activity of the process of glycolysis in them, which serves as a source of energy [20,23]. In order to establish how anaerobic transformation of carbohydrates functions in cardiomyocytes under such conditions, we studied the activity of the glycolitic enzyme, aldolase. The obtained data are presented in **Fig. 1(E)**. The activity of aldolase under the conditions of the studied stress was diminished by ~40% in comparison with the control data.

Thus, as it can be shaped from the obtained results, isolation and disruption of circadian rhythm represent the factor that causes diminution of energy metabolism in cardiomyocytes accompanied by oxidative stress, which, in its turn, can be the cause for appearance of toxic radicals. Long duration of stress can cause certain irreversible changes, for instance, antioxidant system of the cells may be deviated so violently out of the norm that it will be followed by an irreversible pathology of the cardiovascular system.

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 [27–29]. 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 [30].

It is known that quantitative changes in NO are responsible for the formation of various pathologies and also initiate the process of LPO [31]. The primary target of the reactive forms of oxygen resulting from these processes is the mitochondrial respiratory chain and various mitochondrial iron-sulfurous proteins, followed by alteration of mitochondrial metabolism and energy deficiency of cells. This explains why almost 70% deficiency of the aconitase activity in cardiomyocytes can be regarded as a marker specific to oxidative stress [32] [**Fig. 1(A)**]. The causes of energy deficiency also included reduction in the activity of succinate dehydrogenase and creatine kinase due to isolation and disruption of the circadian rhythm [**Fig. 1(C,D)**]. Decrease in the activity of citric acid cycle (aconitase, succinate dehydrogenase) and, in general that of complexes of mitochondrial dehydrogenases are associated with a broad

range of cardiovascular pathologies. The intensity of the process of glycolysis was also diminished [**Fig. 1(E)**]. The obtained results imply that cardiomyocytes of animals under stress suffer rather considerable energy deficiency, which ultimately has an impact on their functional state. Increased quantities of lipids in blood also caused the same effect (**Table 2**). Thus, processes taking place under psycho-emotional stress can serve as causative factors with regard to various pathologies of the cardiovascular system.

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.) [28,32]. 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, cardiomyopathy, 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·), 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 1**), 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 3**) will damage endothelial cells and cause decrease in secretion of NO. This is followed by deepening of endothelial dysfunction, which is revealed in vasoconstriction, hypocoagulation, and proliferation of smooth muscle cells. At the same time, free radicals can damage cardiomyocytes 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 [11,33].

In case of social isolation of animals, decrease in the activity of antioxidant enzymes SOD and calatase in blood as well as in cardiomyocytes 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 cardiomyocyte 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 [34]. Thus, isolation of the animals and disruption of their circadian rhythm represent a stress factor that causes biochemical transformations in blood and cardiomyocytes that leads to a broad range of pathologic conditions of the cardiovascular system.

Apart from the processes described above, changes in many other metabolic processes also take places, which are vital for normal functionality of an organism. It has been found that stress results in quantitative changes in some hormones, such as glucocorticoid, catecholamin and melatonin [35,36]. Therefore, the objective of our current research is to establish the quantitative changes of the hormones under psycho-emotional stress, such as isolation and violation of circadian rhythm.

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