



The Level of Reactive Carbonyl Derivatives of Proteins, Methylglyoxal, and Malondialdehyde in Rats Experiencing Chronic Unpredictable Moderate Stress

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Abstract

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BACKGROUND: Prolonged exposure to stress is known to change homeostasis and can lead to the development of psychopathological disorders, including depression. Exposure to stress factors contributes to the emergence of reactive oxygen species in the brain. The data characterizing changes of the oxidative metabolism under conditions of chronic stress are insufficient, which determine the relevance of the research in this direction. The determination of reactive carbonyl derivatives of proteins (RCDP), methylglyoxal (MG), and malondialdehyde (MDA) levels can be considered an informative method for studying the dynamics of oxidative metabolism.

AIM: The aim of the study was to study the RCDP, MG, and MDA in red blood cells and blood plasma in rats experiencing chronic unpredictable moderate stress (CUMS).

METHODS: A total of 20 male outbred rats weighing 450–500 g were used in the study. The animals were divided into two groups of ten rats each using randomized selection. The experimental group of animals was exposed to the impact of diverse stress factors, according to the CUMS model for 21 days. The control group of animals remained in standard conditions and did not undergo CUMS model. Blood sampling was performed twice for all rats the day before the experiment started and on 21st day to determine the level of RCDP, MG, and MDA in their erythrocytes and blood plasma.

RESULTS: The study revealed a significant decrease of RCDP ($p = 0.007$) and MDA levels in erythrocytes ($p = 0.013$), and MDA level in blood plasma ($p = 0.005$) of rats of the main experimental group. The control group showed a significant increase of RCDP level in plasma ($p = 0.008$), a two-fold increase of MDA in erythrocytes ($p = 0.05$) with a decrease of MDA in plasma ($p = 0.011$). A tendency for MG increase was noted in erythrocytes of rats in both the main group and the control group.

CONCLUSION: The early stages of CUMS were accompanied by the violation of oxidative metabolism with the emergence and accumulation of RCDP and MDA in the blood of animals. The decreased emergence of RCDP and MDA in the later stages can be associated with an excess of oxidized substrates in the blood of animals of the experimental group. The revealed trend toward MG growth in animals of both groups suggests its increased synthesis in erythrocytes, which, in our opinion, should be considered a negative factor affecting the deformability of red cells. In turn, a violation of the deformability of red blood cells leads to a violation of microcapillary blood flow, which contributes to the development of hypoxic damage in different tissues. Thus, the obtained data confirm the different directions of changes in RCDP and MDA levels in the blood of animals experiencing CUMS or acute stress. Our results prove the need for further research on changes in oxidative metabolism in chronic stress disorders.

Introduction

Stress is considered one of the important causes of somatic and mental pathology, including adaptation disorders [1], [2]. Enough data have been obtained confirming the direct relation of stress with the development of depression [3], [4]. Stress changes homeostasis, affects the neuroendocrine and immune systems, and leads to the evolvment of psychopathological syndromes [5], [6].

Reactions to acute stress (AS) and chronic stress suggest various biochemical changes and subsequent behavioral disorders. It has been established that under the influence of stress factors the level of reactive oxygen species in the brain increases [7], [8]. Separate sporadic studies demonstrate certain differences in the direction of the oxidative metabolism reactions in tissues during acute and chronic stress [9].

The mechanism of influence of chronic stress is of greatest interest to researchers. Chronic stress is known to trigger the release mechanism for a significant

number of neurotransmitters with the formation of a pro-inflammatory response of microglia and to cause structural changes in neuroglia [10], [11], [12]. Chronic stress alters the expression of genes that regulate antioxidant enzymes such as catalase, glutathione peroxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase, and glutathione reductase [13], [14], [15]. Activation of lipid peroxidation and inhibition of the mitochondrial respiratory chain are shown in the model of chronic immobilization stress [16]. A study of patients with depression and bipolar disorder revealed a decrease in antioxidant protection and an increase in the level of lipid peroxidation in blood [17], [18]. A new meta-analysis confirmed an increased level of 8-hydroxy-2'-deoxyguanosine and F2-isoprostanes in tissues and biological fluids, marking increased oxidative damage to DNA and lipids in patients with bipolar disorder and various types of depression [19].

Changes in oxidative metabolism under the influence of chronic stress are an important aspect of understanding its damaging effects. Studies show changes in levels of oxidative stress markers in mental and neurological pathologies. An increase in the level of malondialdehyde (MDA) and the number of carbonyl groups is recorded in cerebral ischemia [20]. The damaging effect of methylglyoxal (MG) is mediated by the formation of MG-modified proteins with the development of carbonyl stress in neuroblastomas [21]. The process of inactivation of proteins by the oxygen active species is a direct factor in the development of carbonyl stress with the succeeding progression of neurodegenerative disorders [22], [23], [24]. A significant number of researchers conducted studies of isolated indicators of carbonyl stress or its end products in the already evolved pathological conditions. However, according to available data, exposure to chronic stress leads to activation of oxidative stress mechanisms with stimulation of the "hypothalamus–pituitary–adrenal gland" chain reaction with ensuing systemic damage [25]. Thus, the study of neuron-specific markers and isolated fractions of carbonyl proteins in our study were not justified, while reactive carbonyl derivatives of proteins (RCDP), MG, and MDA could be considered the most informative indicators of oxidative stress and can be used as markers of oxidative damage at the stage of pathology formation and as a control for further therapy.

An analysis of the literature data showed, that markers of the state of oxidative metabolism, such as RCDP, MG, and MDA, have hardly been studied under conditions of chronic stress, which explains the relevance of this study.

Protein carbonylation is a common protein modification mechanism. Carbonyl derivatives can appear in different ways: As a result of direct oxidation of the amino acid residues of lysine, arginine, proline and threonine, or under the action of reactive oxygen species, as well as in reactions with carbonyl-containing oxidized lipids. Other carbonylation pathways include

protein fragmentation, α -amidation, and oxidation of glutamine side chains [26]. Protein carbonylation is an irreversible process leading to a loss of proteins' functions. Under physiological conditions carbonylation plays an important role in protein quality control and protein metabolism. Increased carbonylation leads to accumulation of proteolysis-resistant aggregates of oxidized proteins [27]. Some of neurodegenerative diseases are associated with the accumulation of carbonylated protein aggregates in cells and tissues [28].

MG appears mainly in the process of glycolysis from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The utilization of MG into D-lactate occurs through the glyoxylase system with the participation of reduced glutathione. The pentose phosphate pathway is also involved in the metabolism of MG as a source of NADPH, which is necessary for the reduction of oxidized glutathione. MG is a highly reactive dicarbonyl, capable of forming adducts with proteins and nucleic acids, leading to the loss of their biological functions [29].

MDA is the product of arachidonic acid and other polyunsaturated fatty acids decomposition and appears as a result of enzymatic and non-enzymatic reactions. The functions of MDA are currently under active study. It is known that MDA can work as a signal messenger, as well as regulate the expression of a number of genes. On the other hand, MDA is able to form adducts with proteins and DNA, which leads to disruption of their structure and loss of biological functions [30].

The aim of our study is to examine changes in the levels of RCDP, MG, and MDA in red blood cells and blood plasma in rats experiencing chronic unpredictable moderate stress (CUMS).

Methods

To reproduce the conditions of chronic stress, a model that met the criteria of credibility and reliability was selected. In recent years, the model of "CUMS" has confirmed its position as a reference model for the study of the neurobiological basis of stress-induced disorders and the development of new antidepressants [31], [32], [33].

Selection and description of participants

A total of 20 male outbred rats weighing 450–500 g. were used in the study. The animals were kept (five rats per cage) under standard laboratory conditions ($25 \pm 2^\circ\text{C}$, humidity 60–70%), maintaining a 12-h natural day-night cycle with free access to standard food and water. The quarantine and keeping conditions of the animals before the start of the experiment met

the established requirements. The rats were divided into two groups ten animals each using randomized selection. The study was conducted in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals Used in Experiments and Other Scientific Purposes (Strasbourg, 1986), requirements of GLP OECD, EAEU Rules of Good Laboratory Practice No. 81, order of the Ministry of Health and Social Development of the Republic of Kazakhstan No. 392 dated May 25, 2015. The study was approved by the decision of the Committee on Bioethics of the Karaganda Medical University on June 17, 2019, protocol No. 65.

Technical information

The experimental group of animals was exposed to the CUMS, while the comparison control group was kept in standard conditions without CUMS. Animals of the two groups underwent behavioral testing and blood sampling procedure. Thus, the animals of the control group can be considered an animal model of AS, which was caused in animals 4 times during the study period.

Behavioral assessments

Animals of the two groups were tested according to the standard protocols in the “open field test” [34], “the elevated cross-maze test” [35], “tail-hanging test” [36], [37], and “sucrose preference test” [38]. Behavioral tests were performed on the day preceding the start of the experiment, designated as first control point, and at the end of each cycle of the CUMS model, which corresponded to the 7th, 14th, and 21st days of the experiment. The day of the behavioral tests additional stress factors were absent, since the animals were placed into the new environment for the behavioral testing and that can be considered as a stressful factor itself. A rest period of 1 h was allowed in between each trial.

Animals in two groups underwent the blood sampling from the tail vein, blood being gathered into heparinized tubes. The procedure was performed under light ether anesthesia on the day preceding the start of the experiment, designated as first control point, and on the 21st day of the experiment, designated as a second control point. Blood sampling in animals was carried out after behavioral testing, since the blood sampling procedure and the effects of ether anesthesia could have affected the results of behavioral tests and had been a significant stress factor.

Experimental procedure for CUMS model

For 21 days, the animals of the main experimental group were experiencing the trials

according to the “CUMS” model, presented in Table 1 (three cycles of 7 days each).

Table 1: Exposure schedule for rats of the main group, leading to formation of the chronic unpredictable moderate stress model

Type of exposure	Day of experiment cycle						Control test
	1	2	3	4	5	6	
Narrow cell	12.00–08.00						Behavioral tests
Food deprivation	8.00–08.00						
Immobilization in a case	10.00–14.00						
Shading during daylight hours	6.00–21.00						
Water deprivation	8.00–18.00						
Artificial light during night hours	19.00–10.00						
Full immobilization	10.00–12.00						

Types of exposure used to form the CUMS model:

- Narrow cell – limiting the space of the cell by half due to the installation of an additional partition in the cell
- Food deprivation – food deprivation for 24 h
- Immobilization in a case – animals are placed in individual cases, which do not deprive them of the ability to move, but greatly limit the space for movement
- Shading during daylight hours – artificial dimming during daylight hours, lasting 15 h
- Water deprivation – deprivation of water for 10 h
- Artificial light during night hours – creating artificial lighting in the nighttime hours, lasting 15 h
- Full immobilization – placing animals in individual canisters, depriving them of the ability to move for 2 h.

Spectrophotometric study

Blood samples obtained during the study were transferred to the laboratory, where they underwent centrifugation and analysis of the collected materials by spectrophotometry using a PD-303 ultraviolet (UV) digital UV-VIS spectrophotometer, manufactured by APEL, Japan.

Determining RCDP

We measured the concentration of RCDP following the well-tested protocol of Levine *et al.* [39], [40]. To triplicate aliquots of plasma (0.8 mL), we added 0.2 mL of 10% trichloroacetic acid solution and centrifuged the samples. Then, either 1 mL of 2M HCl liquid solution or 10 mL of 2.4-dinitrophenylhydrazine (DNPH) in 2M HCl solution was added to the precipitates, and further this mixture was incubated at 37°C for 90 min. Next, the samples were centrifuged again (8000 rpm, 10 min) and the DNPH excess was removed with ethanol-ethyl acetate 1:1 (v/v). Finally, the samples were re-suspended

in 6M of guanidine hydrochloride. Quantification was performed using a spectrophotometer PD - 303 UV APEL (Japan) at a 366 nm absorbance. Concentration of carbonyl derivatives was calculated using the molar absorption coefficient of 22,000 mol/cm⁻¹, RCDP values were given in nmol/ml.

Determining MGs

MG was determined by reaction with dinitrophenylhydrazine according to a modified Racker method [41]. We combined 25 µl of blood sample with 350 µl of DNPH (0.1% DNPH in 2N HCl) and then added 2.125 mL of distilled water to each tube. Finally, we incubated samples for 15 min at 37°C and after the incubation added 1.5 mL of 10% NaOH. The absorbance for spectrophotometer was set at 576 nm; MG levels were expressed in percent absorbance of MG.

Determining MDA

MDA in red blood cells was determined according to the protocol of Goncharenko and Latypova [42]. To determine MDA, erythrocytes were washed in 10 mL of chilled saline, and then centrifuged for 10 min at 3000 rpm. The washed red blood cells were hemolyzed with distilled water with a 1:5 ratio. For the study, we took 0.3 mL of hemolysate, added 0.3 mL of 10% phosphotungstic acid and 10 min later centrifuged the samples at 3000 rpm for 10 min. The resulting precipitate was washed with 1 mL of distilled water and centrifuged once again. Then, 3 mL of water and 1 mL of thiobarbituric acid (80 mg of tert-butyl alcohol in a mixture of 5 mL of water and 5 mL of acetic acid) were added to the precipitate and incubated for 60 min in a boiling water bath. Finally, the solution was centrifuged and tested for absorbance at spectrophotometer set at 532 nm against water. In the calculations, we used the molar extinction coefficient, which is 1.56 × 10⁵M/cm⁻¹ expressed in µmol/mL.

Statistics

The results were statistically processed using the SPSS.Statistics.v22. multilingual-EQUiNOX (SPSS Inc.) software.

Analysis of the obtained data was conducted using descriptive statistics. The comparison of oxidized metabolism before and after the experiment was carried out using the non-parametric Wilcoxon t-test (α = 0.05).

Results

To compare the studied parameters in blood plasma and erythrocytes on the first and second control

points (a day before the experiment and 21st day of the experiment), the non-parametric Wilcoxon t-test (α = 0.05) was used, where statistically significant differences were accepted at p ≤ 0.05.

The results of studies in the main group, presented in Table 2, show a reliably significant decrease of RCDP level in erythrocytes by 1.67 times (median, p = 0.007), MDA level in plasma (p = 0.005), and MDA level in erythrocytes (p = 0.013) on the 21st day of the experiment. A tendency toward a decrease in RCDP level in blood plasma and MG level in erythrocytes of rats of the main group was also revealed.

Table 2: RCDP, MDA levels in plasma and RCDP, MDA, MG levels in erythrocytes in the main group

Indicator	Median (Q25; Q75) before the experiment	Median (Q25; Q75) after the experiment	p
RCDP in erythrocytes	10,74 (9,498;12,81)	6,403 (3,939;8,866)	0,007
MDA in erythrocytes	10,00 (8,846;10,90)	9,231 (8,077;19,49)	0,013
MG in erythrocytes	0,615 (0,573;0,639)	0,757 (0,728;0,828)	0,445
RCDP in plasma	1,052 (0,857;1,134)	0,996 (0,848;1,212)	0,878
MDA in plasma	1,720 (1,694;1,774)	1,548 (1,350;1,668)	0,005

MG: Methylglyoxal, RCDP: Reactive carbonyl derivatives of proteins, MDA: Malondialdehyde.

Table 3 shows the results of a study of the target parameters in the blood of rats of the comparison group. On the 21st day of the experiment, there was a significant increase of RCDP level in plasma (p = 0.008) and MDA in erythrocytes twice from the initial level (p = 0.05), while MDA in plasma decreased (p = 0.011). There also was a tendency toward an increase of RCDP and MG levels in the erythrocytes of rats of the comparison group.

Table 3: RCDP, MDA levels in plasma and RCDP, MDA, MG levels in erythrocytes in the comparison group

Indicator	Median (Q25; Q75) before the experiment	Median (Q25; Q75) after the experiment	p
RCDP in erythrocytes	1.983 (1.792;2.121)	2.424 (1.662;3.013)	0.441
MDA in erythrocytes	6.795 (5.769;8.077)	13.97 (8.718;21.54)	0.050
MG in erythrocytes	0.787 (0.758;0.897)	0.807 (0.676;0.831)	0.575
RCDP in plasma	0.628 (0.606;0.753)	1.377 (1.117;1.411)	0.008
MDA in plasma	2.250 (1.694;2.833)	1.482 (1.376;1.508)	0.011

MG: Methylglyoxal, RCDP: Reactive carbonyl derivatives of proteins, MDA: Malondialdehyde.

The results of the study show that the levels of RCDP and MDA are reduced in blood plasma and in erythrocytes of the animals experiencing CUMS. At the same time, in animals with AS the revealed changes in the studied parameters have the opposite direction, which are documented by an increase in the content of RCDP in blood plasma and erythrocytes and MDA in erythrocytes. At the same time, a decrease of MDA level in the blood plasma of animals with an AS factors was recorded. In other words, we found that oxidative stress markers levels change in opposite directions depending on the type of stress factors exposure.

Discussion

According to the generally accepted theory of the effect of stress on the body, the development

of stress-induced disorders directly depends on the adaptation mechanisms, duration, and strength of the stress factor influence and neurobiological vulnerability to behavioral disorders [32]. Available studies indicate that each stress model has a different effect on the tissues of various organs [43]. These assumptions are confirmed in our study. The data obtained from the study of animals in the model of chronic stress show predominant damage in red blood cells, which may explain, among other things, damage to the central nervous system with the formation of stress-induced disorders. The study of the mechanisms of the stress, affecting biochemical processes through a change in the oxidative modification of proteins and lipid peroxidation, still continues.

In our experiment, the CUMS and AS models were used to form chronic and AS-induced disorders. The CUMS model implies daily exposure to unpredictable stress factors of small strength. In addition, behavioral reactions were studied in animals of this group.

In the AS model, the animals were not subjected to daily stress exposure of low strength, but they were tested in behavioral tests. The behavioral assessment procedure included a "tail suspension test," which in itself can be considered as a rather traumatic, if AS factor [44].

Our results of assessing the RCDP and MDA levels allowed us to conclude that the development of carbonyl stress in the blood and the activation of lipid peroxidation in rat blood with a model of AS happen simultaneously. At the same time, a decrease of RCDP and MDA levels was observed on the 21st day of the experiment in the blood of rats experiencing CUMS. In our opinion, the following assumption can be made as a hypothesis: In all likelihood, the early stages of CUMS were accompanied by the violation of oxidative metabolism with the emergence and accumulation of RCDP and MDA in the blood of animals. The decreased emergence of RCDP and MDA in the later stages can be associated with an excess of oxidized substrates in the blood of animals of the experimental group. The revealed trend toward MG growth in animals of both groups suggests its increased synthesis in erythrocytes, which, in our opinion, should be considered a negative factor affecting the deformability of red cells [45]. In turn, a violation of the deformability of red blood cells leads to a violation of microcapillary blood flow, which contributes to the development of hypoxic damage in different tissues.

When comparing the results in the main group and in the comparison group, various trends of changes after the experiment were revealed. Hence, the concentration of RCPD in the plasma and erythrocytes of rats of the main group showed a pronounced increase after the experiment (by 111% and 77%, respectively). In the comparison group, such changes were not observed: There was no significant growth in plasma

RCPD level and, contrariwise, even a decrease in the concentration of RCPD by 41% in red blood cells was noted.

Changes in the concentration of MDA in blood plasma and red blood cells were unidirectional in both groups, but with varying degrees of severity. Hence, in the red blood cells of rats of the main group the concentration of MDA after the experiment increased by 177%, while in the comparison group there was only an increase by 25%. In the blood plasma of rats of the main group a decrease in the concentration of MDA by 40% was noted, while in the comparison group the decrease in concentration was only at the 15% level. Changes in the concentration of MG in the blood of rats of the main group were not pronounced, while in the comparison group the concentration increased by 28%. The revealed differences in the results dynamics in the groups demonstrate involvement of the separate oxidative metabolism elements in the various pathological mechanisms of acute and chronic stress formation. According to the results of the obtained data, the predominant accumulation of oxidative metabolites in red blood cells was revealed, which is a factor of subsequent hypoxic tissue damage. The changes in the MG level which does not correlate with the levels of RCDP and MDA, in our opinion, can be explained by its predominant accumulation in tissues, where under the conditions of oxidative stress it mediates the maximum damaging effect. This assumption requires further study of tissue indicators of MG concentration. A significant increase in the level of MDA in red blood cells in the main group, in our opinion, may be a cause of the early death of erythrocytes and the loss of their biological functions due to the high degree of toxicity of the MDA molecule [46]. The accumulation of RCDP in turn contributes to inhibition of mitochondrial respiration function with the development of ensuing tissue hypoxia.

Conclusion

Thus, the obtained data confirm the different directions of changes in RCDP and MDA levels in the blood of animals experiencing CUMS and AS. Our results prove the need for further research on changes in oxidative metabolism in chronic stress disorders.

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