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Glyoxal and Methylglyoxal Ultra-Low Doses Activate Lymphocytes Energy and Nitrogen Metabolism in *Vitro*.

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ABSTRACT

For the first time it was investigated the effect of ultra-low doses of glyoxal (1.29×10^{-12} M) and methylglyoxal (1.04×10^{-12} M) on the separate links of energy and nitrogen metabolism in lymphocytes *in vitro* at a different time points during72 hours exposure (3, 6, 24, 48 and 72 hours). It was shown that active carbonyl compounds caused significant changes of aldolase, lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase activities and concentrations of pyruvate and extracellular glucose content. It was detected the opposite direction of the glucose utilization speed at the study and control lymphocytes culture. We discovered activation of energy exchange under 3 and 24-hour exposure to ultra-low dose of glyoxal and methylglyoxal.
Keywords: ultra-low concentration, active carbonyl compounds, aldolase, lactate dehydrogenase, alanine aminotransferase, pyruvate, glucose.

Abbreviations: ACS, *active carbonyl species;* ALT, *alanine aminotransferase;* AST, *aspartate aminotransferase;* GL, *glyoxal;* LDH, *lactate dehydrogenase;* MGL, *methylglyoxal.*

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INTRODUCTION

The study of various aspects of the metabolism of active carbonyl species (ACS) is an important task of modern biology. The most well-known of them are glyoxal and methylglyoxal. These compounds are permanently present in the normally functioning cells as the main and by-products of the different metabolic pathways: lipid and protein peroxidation, non-enzymatic oxidation of some amino acids and others. The concentrations of the GL and methylglyoxal in the body are in the range of 1×10^{-7} M-1×10^{-8} M [1].

At high concentrations (greater than 1×10^{-7} M), glyoxal and methylglyoxal are cytotoxic compounds, with life expectancy up to one hour. They can glycolysis proteins, lipids and nucleic acids, disrupting their function [2]. In the cell, there are several mechanisms of inactivation glyoxal and methylglyoxal capable of supporting the concentration of such ACS at a level that is close to the physiological state. The most important role in this process plays a glutathione system. As a result, normally functioning organism has a constant dynamic balance between the formation and degradation of ACS, but which can be altered by different factors [1, 2, 3, 4,5]. Thus, the development of much pathologies of various genesis, as well as influence of adverse exogenous factors, including ionizing radiation, can lead to accumulation of glyoxal and methylglyoxal [6]. Their intracellular concentration increases, which leads to the structural and functional disorders of the body. This process was named carbonyl stress[2,5].

High concentrations of glyoxal and methylglyoxal make their contribution to the development of atherosclerotic vascular damage, the appearance of microangiopathies, nephropathy, and neuropathy. They stimulate the secretion of the inflammatory cytokine, endothelial growth factor expression and much more [1,4].Carbonyl stress complicates the progress of cancer, the diabetes mellitus type I and II, cataracts, Alzheimer's disease and others [1,3,4,5].

At present, apart from pathological influence glyoxal and methylglyoxal under carbonyl stress, biologists are increasingly studying the functions of these highly-reactive compounds in various physiological processes [5]. In particular, it was studied the important role of glyoxal and methylglyoxal in thrombocytes aggregation, monocytes chemotaxis, cell signaling, gene expression regulation, immune response, energy metabolism[7]. It was proven the participation of glyoxal and methylglyoxal in the defending against the pathogenic microflora, viruses and cancer cells [8]. A. Akhand et al., (2001) showed that glyoxal and methylglyoxal initiated MAPK-signaling pathways in human endothelial cell culture, indicating that these ACS were acting in the intracellular messaging [9]. Study by Bhattacharyya et al., (2008) was described that methylglyoxal can enhance the non-specific immunity of the host against tumor cells. Methylglyoxal increased the number of macrophages in the peritoneal cavity of both normal and tumor-bearing mice. It also elevated the phagocytic capacity of macrophages in both these groups of animals. Methylglyoxal also played a role in the proliferation and cytotoxicity of splenic lymphocytes[10].

There are ongoing attempts to use high (much above physiological) concentrations of this ACS for therapeutic purposes[3,11,12]. Since glyoxal and methylglyoxal could be called highly-reactive intracellular compounds that play an important role in the life of organism it could be expected that they affect at ultra-low doses[11]. But these effects of exogenous glyoxal and methylglyoxal on various intracellular processes remain unclear as well as the mechanisms of their action. However, given into account the structural and functional features of the glyoxal and methylglyoxal, it is natural to wait for a variety of effects at the metabolic links under action by sub-physiological concentrations of these compounds (less than $1 \cdot 10^{-8}$ M).

The aim of the present study was to investigate the influence of ultra- low doses of glyoxal and methylglyoxal on some links of the lymphocytes energy and nitrogen exchange *in vitro.*

MATERIALS AND METHODS

Study Participants

We recruited 16 healthy adult volunteers in “Cascade-Medical Reference Laboratory UBI”, Glevakha, Ukraine (8 men and 8 women). A healthcare professional (nurse or physician) conducted a verbal review of clinical history to determine eligibility based on the absence of any major chronic illness, current medication administration or symptoms of infection. Blood samples were collected in accordance with the guidelines of

the Declaration of Helsinki (2013) of the World Medical Association. Each individual gave written informed consent to participation. The median age of all recruits was 40.1 years old.

Cell isolation and culture condition

Lymphocytes were obtained through the collection of human peripheral blood by venipuncture procedure in vacuum/siliconized tubes containing 0.1 mM EDTA. Obtaining lymphocytes from donor blood was carried out by gradient density sedimentation on Histopaque-1077 Hybri-Max® (Sigma, USA), the density of 1.077±0.001 g/ml [12]. Before gradient density sedimentation blood with an anticoagulant was diluted with sterile PBS without Ca^{2+} and Mg^{2+} (Sigma, USA) 1: 1.25. Centrifugation was performed at 400g for 30 minutes. Received mononuclear fraction was washed for three times with sterile PBS without Ca^{2+} and Mg^{2+}. Lymphocytes were isolated using mononuclear adhesion to plastic (mononuclear cells were incubated in Petri dish for 30 minutes). It was counted cells in lymphocytic fraction and assessed their viability in the Goryaev's chamber by trypan blue. Lymphocytes pool of one donor was divided into three equal parts, which further investigate as control and experimental cultures.

Lymphocytes incubated in the medium RPMI-1640, contained 5% bovine fetal serum, 40 mML-glutamine; 10 thousand units penicillin and 10 mg streptomycin (all reagents by Sigma, USA) in 100 ml of medium in sterile Petri dish (Sarstedt AG & Co, Germany) at 37°C, 5% CO_2, and humidity 95%. The final lymphocytes concentration in the control and experimental cultures was 3×10^6/ml. After the 36-hours incubation period, experimental lymphocytes cultures were treated by glyoxal and methylglyoxal in 0.9% NaCl solution (GlyoxalCompositum ®, «Heel», Germany). The final concentration of glyoxal and methylglyoxal were 1.29×10^{-12}M and 1.04×10^{-12}M, respectively, which named extremely low [13]. At the same time the control cultures were treated with the relevant volumes of 0.9% NaCl.

Before the glyoxal and methylglyoxal/ or 0,9% NaCl treatment and after finishing of the 3, 6, 24, 48 and 72 hours cycles it was received culture medium probes and lymphocytes suspensions (by three times washing in PBS without Ca^{2+} and Mg^{2+} after cultural medium with followed centrifugation at 1200g for 10 minutes). At the next stage, it was received samples (lysates) by three times freezing and thawing.

It was determined an activity of key enzymes of nitrogen metabolism alanine aminotransferase (EC 2.6.1.2) (ALT), aspartate aminotransferase (EC 2.6.1.1) (AST) in lymphocytes (both by ELItech, France). The intensity of glycolysis was evaluated by the activity of lactate dehydrogenase (EC 1.1.1.27) (LDH) (ELItech, France) and aldolase (EC 4.1.2.13) (Sentinel Diagnostics, Italy)[14], and by the pyruvic acid content (NPO Abris+, Russia) in lymphocytes, along with the changes of glucose concentration in the culture medium. All lysates were tested for protein content by Lowry protein assay. All measurements were made by biochemical analyzer Microlab 300, Vital Scientific (Netherlands). Key enzyme activity was expressed in U/L*g protein, a concentration of glucose and pyruvic acid in mg/dL*g protein and mmol/L*g protein, respectively.

Statistical analysis

To assess statistically significant changes experimental samples compared to control was used a nonparametric criterion of marks G. To assess the nature and strength of relationships between the control and experimental groups was used pair correlation r-index. For all analysis statistical significance was set at $p<0.05$ [15].

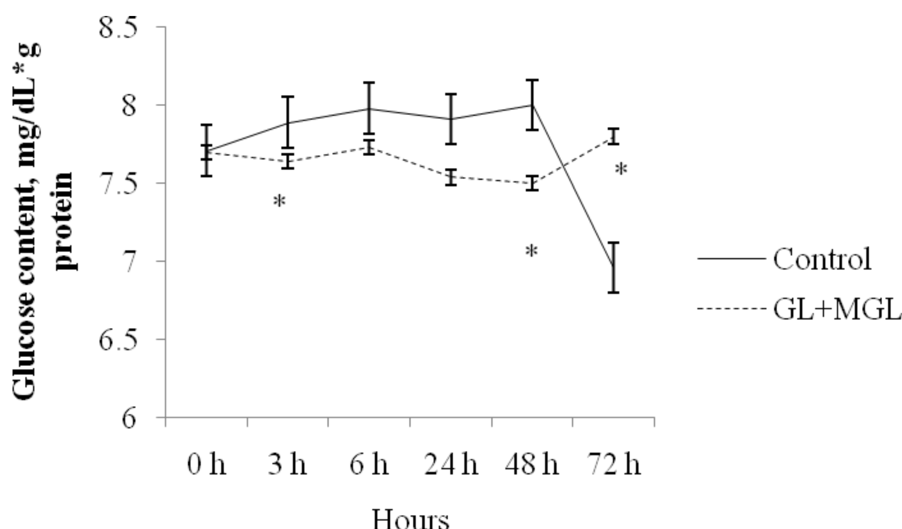
RESULTS

The time-course effect of glyoxal and methylglyoxal ultra-low doses on the culture medium glucose content in different time points: 3, 6, 24, 48 and 72 hours

In the 72-hours *in vitro* experiment it was studied the influence of the ultra-low concentrations of glyoxal and methylglyoxal on the lymphocytes energy and nitrogen metabolism. There was studied the intracellular activity of key enzymes and the most important metabolites concentration which took a part in such processes.

The experiments revealed significant changes in glucose concentration in the culture medium. Thus, the significant decreasing of an extracellular content of glycolysis substrate was observed after 3 hours from the beginning of glyoxal and methylglyoxal action in ultra-low concentrations (Fig.1). While the 6- and 24-hours lymphocytes cultivation with these ACS didn't lead to significant changes in glucose concentration. However, within 48 hours from the beginning of action by the ultra-low concentration of glyoxal and methylglyoxal on lymphocytes significant 1.1-fold decreasing of the extracellular glucose content was identified. At the final stage of the experiment (72 hours) in the culture medium it was shown the increase of glucose concentration by 12% ($p<0.05$) (Fig.1).

Figure 1: Changes of glucose content (mg/dL*g protein) under ultra-low doses of glyoxal and methylglyoxal (1.29×10-12M and 1.04×10-12 M, respectively) influence on lymphocytes during different time points: 3, 6, 24, 48 and 72 hours,(M±SE, n=16).



*- $p<0.05$ – differences between control and GL+MGL groups.

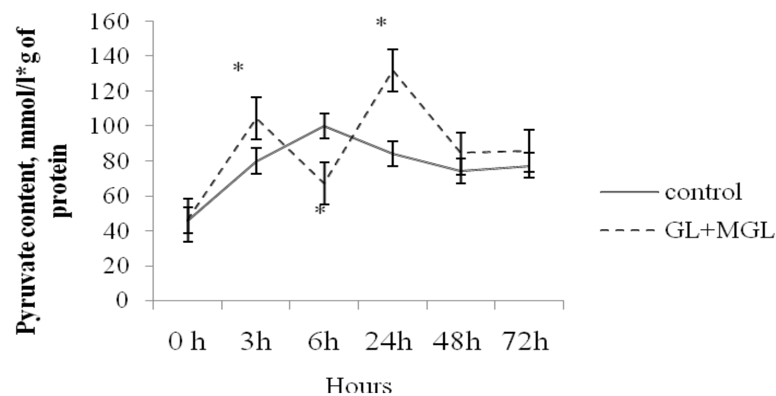
In control group was admitted non-significant increasing of the glucose content in culture medium whereas in studied group methylglyoxal with glyoxal induced increasing of glucose utilization by lymphocytes cells. Correlation analysis found an inverse relationship ($r= -0.67$, $p <0.05$) between the control and experimental extracellular glucose content during the experiment.

The time-course effect of glyoxal and methylglyoxal ultra-low doses on the pyruvic acid content for 3, 6, 24, 48 and 72 hours influence on the lymphocytes cells.

It was found the increase of intracellular concentration one of the key metabolites of energy exchange, pyruvic acid, by 30% ($p<0.05$) under 3-hours exposure data (Fig.2). Such changes may be the result of medium substrates oxidation (glucose, amino acids) by lymphocytes. However, the 6-hours effect on lymphocytes by selected ACS led to the significant decrease of pyruvic acid content in test samples by 33% ($p<0.05$). More significant changes in the content of pyruvic acid were detected after 24 hours incubation in the ultra-low concentrations of glyoxal and methylglyoxal: the level in the experimental samples was 1.6-fold higher than control ($p<0.05$).

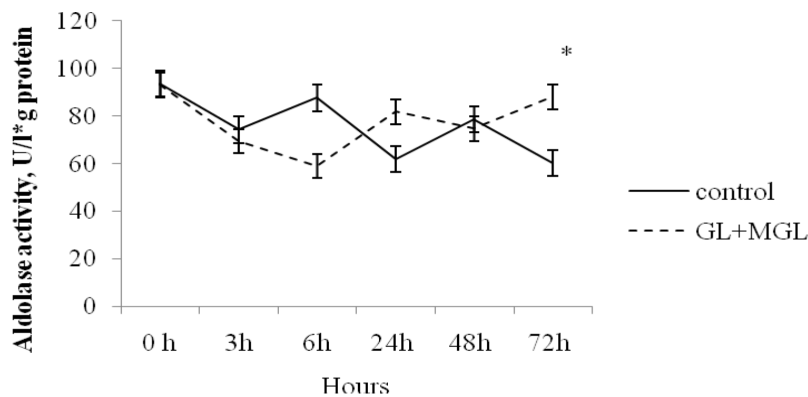
The 48- and 72-hours ultra-low effects of ACS didn't accompany by significant changes of lymphocytes pyruvic acid content (Fig.2). It was admitted that such effect of glyoxal and methylglyoxal was accompanied by modulations in the glucose content (Fig.1): in control group the rather stable level of glucose content was in accordance with the stable level of pyruvic acid, while under the ACS action, decreasing in glucose culture content was accompanied by relative increasing of the pyruvic acid formation (Fig.1 and 2). It could indicate the same intensity of the formation and use of this metabolite in intracellular respiration in control group and its intensifying under ACS's action.

Figure 2: Changes of pyruvic acid content (mmol/l*g protein) under ultra-low doses of glyoxal and methylglyoxal (1.29×10-12M and 1.04×10-12 M, respectively) influence on lymphocytes during different time points: 3, 6, 24, 48 and 72 hours, (M±SE, n=16).



*- p<0.05 – differences between control and GL+MGL groups.

Figure 3: Changes of aldolase activity (U/l*g protein) under ultra-low doses influence of glyoxal and methylglyoxal (1.29×10-12M and 1.04×10-12 M, respectively) on lymphocytes during different time points: 3, 6, 24, 48 and 72 hours, (M±SE, n=16).



*- p<0.05 – differences between control and GL+MGL groups.

The time-course effect of glyoxal and methylglyoxal ultra-low doses on the aldolase activity for 3, 6, 24, 48 and 72 hours influence on the lymphocytes cells.

As a result of investigations, it was found that the activity of the studied enzymes is less exposed to the impact of ultra-low concentrations of glyoxal and methylglyoxal. This may be due to the action of a complex and multi-level regulatory system of their functioning [16]. The activity of the key enzyme of glycolysis aldolase changed only at the final stage of the experiment, provided 72-hour ultra-low impact by glyoxal and methylglyoxal on lymphocytes. In the experimental samples, it was 1.5-fold higher than the control level (p<0.05) (Fig.3).

The time-course effect of glyoxal and methylglyoxal ultra-low doses on the lactate dehydrogenase activity for 3, 6, 24, 48 and 72 hours influence on the lymphocytes cells.

The level of LDH activity is an informative indicator that characterizes intracellular transformation from pyruvate into lactate. Throughout almost the experiment LDH activity persisted at control levels. Only the 3-hours exposure to ultra-low doses of glyoxal and methylglyoxal led to a significant reduction in LDH activity by 9% (p <0.05) (Fig.4).

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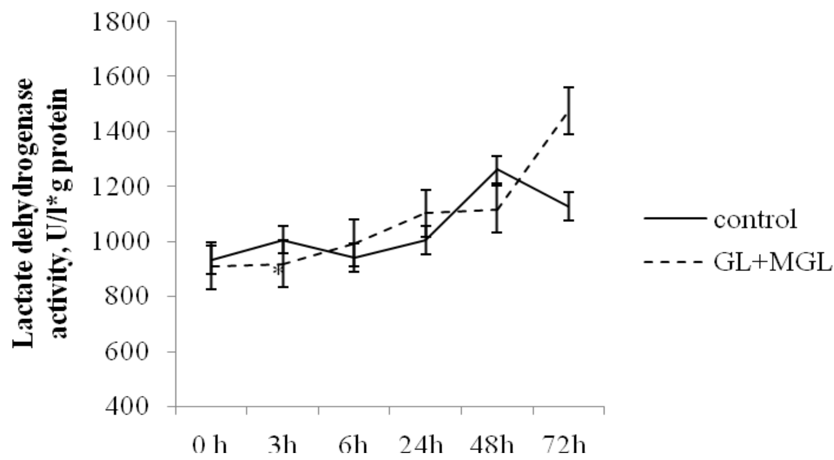
Figure 4: Changes of lactate dehydrogenase activity (U/l*g protein) under ultra-low doses influence of glyoxal and methylglyoxal (1.29×10-12M and 1.04×10-12 M, respectively) on lymphocytes during different time points: 3, 6, 24, 48 and 72 hours, (M±SE, n=16).

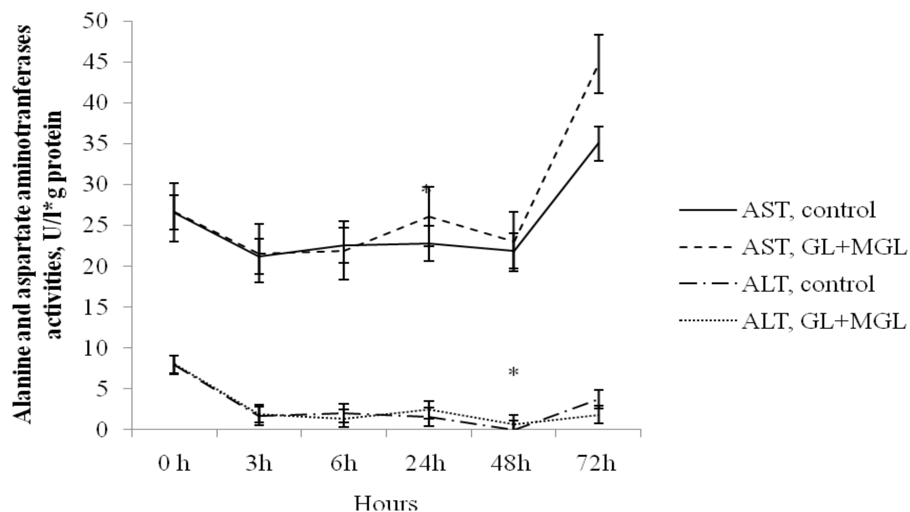


*- p<0.05 – differences between control and GL+MGL groups.

The time-course effect of glyoxal and methylglyoxal ultra-low doses on the aspartate and alanine aminotransferases activity for 3, 6, 24, 48 and 72 hours influence on the lymphocytes cells.

AST and ALT are key enzymes of nitrogen metabolism that are directly related to the internal process of cellular respiration. At the same time, AST and ALT are the main links, providing coordination and communication between protein and carbohydrate metabolism [15,16]. We had found a direct correlation between the activity of AST and ALT in the experimental and control lymphocytes cultures (r=0.97 and r =0.88, respectively, p <0.05). It could testify about no effect of the ultra-low doses of ACS data on nitrogen metabolism of lymphocytes. At the same time, 48-hours ultra-low influence by glyoxal and methylglyoxal in cells induced a significant decrease of ALT activity by 4.4-folds (Fig.5). However, AST activity was significantly higher than controls at 15% (p<0.05) under the 24-hours influence of ultra-low concentrations of glyoxal and methylglyoxal (Fig. 5).

Figure 5: Changes of aspartate and alanine aminotransferases activities (U/l*g protein) under ultra-low doses influence of glyoxal and methylglyoxal (1.29×10-12M and 1.04×10-12 M, respectively) on lymphocytes during different time points: 3, 6, 24, 48 and 72 hours, (M±SE, n=16).



*- p<0.05 – differences between control and GL+MGL groups.

DISSCUSSION

It should be noted currently that there is no any precise theory that would describe the mechanisms of ultra-low doses of biologically active species action on the body or any of its structure. Also, it certainly makes difficult to analysis the results of any studies using ultra-low impact factors of different nature in biological facilities.

Our study had shown that the most significant changes of intracellular energy exchange has appeared under the 3-hour influence of selected substances on lymphocytes. It was shown the most significant decrease in the extracellular glucose concentration at this point of the study (Fig.1). Along with this, an increase in intracellular concentrations of pyruvate testified about the intensification of the glycolytic way of oxidation of the substrate (Fig.1). It should be noted that under these conditions (3-hour exposure) inhibition of LDH (Fig.2) could also contribute to the increased content of pyruvic acid in the studied cultures of lymphocytes. These changes characterizing the energy metabolism were accompanied by lacking significant changes in the transaminase lymphocytes (AST and ALT) activity. These, in turn, could indirectly contribute to increasing the concentration of pyruvate by bringing in energy metabolism amino acids that were the components of the culture medium[18]. It is known that a high content of pyruvic acid activated pyruvate carboxylase complex thereby creating the conditions for the formation of acetyl-CoA - the main substrate of the citric acid cycle [17]. It is, therefore, possible to assume that the 3-hour ultra-low influence on the lymphocytes by glyoxal and methylglyoxal activates not only anaerobic but aerobic energy metabolism also (agreed changes of the glucose and pyruvic acid medium content).

6-hours effect of selected ACS did not cause significant changes in the process of anaerobic respiration and nitrogen metabolism. Almost all indicators that were studied were within the reference values. The level of pyruvic acid is the exception. Its concentration in lymphocytes was significantly lower than in control samples (Fig.1), which may be the result of the intensification of a number of processes: oxidative decarboxylation, the citric acid cycle and oxidative phosphorylation - the basic stage of aerobic respiration. It is also possible to use pyruvate in anabolic processes. It can be spent on the synthesis of amino acids, particular, alanine [18,19]. Despite the reduced intracellular concentration of pyruvic acid, provided a six hours ultra-low effects on cells glyoxal and methylglyoxal, we can conclude the stable intensity of nitrogen and energy exchanges.

The next stage of the experiment (24-hour action selected ACS) found a sharp increase in the concentration of pyruvate and AST activity (Fig.4). These changes in biochemical homeostasis are quite natural. Increased activity of AST ensured inflow of additional quantities oxaloacetate to mitochondria due to its formation from aspartic acid [20]. All this, together with the normal functioning aerobic glycolysis, led to increased pyruvate concentration. It should be noted that at this stage (24-hoursexposure with selected ACS) it was revealed the greatest intracellular concentration of pyruvic acid throughout the experiment, indicating a significant intensification of lymphocytes energy exchange. Naturally, such changes could not affect the rate of energy and nitrogen metabolism for the next stage of the experiment. As is, the results of 48-hour effect on cells of selected ACS reduced ALT activity on the background of a decline in culture medium glucose concentration (Fig.1 and Fig.5). However, other indicators of energy and nitrogen exchange were at the baseline, indicating the normal flow of these processes. In our point of view, just the decreasing of glucose concentration caused the fall of ALT activity, the function of which was to support the physiological level of intracellular alanine pool [18]. In terms of the same deficiency of glucose and extremely saving supply of pyruvate, ALT inhibition is natural.

72-hour exposure of the cells to the selected ACS has led to a significant increase in the concentration of glucose in culture medium (Fig.1). It is possible that under these conditions glucose content increase plays effector function, which is aimed at hexokinetic reactions of glycolysis. As a result, this has led to increased total glycolytic reaction cascade [14, 15] that was expressed in increased aldolase activity (Fig.3).

Heimfarth et al. (2013)in cerebral cortex and hippocampus tissue had shown that high-dose influence1mM methylglyoxal induced increasing production of reactive oxygene species (ROS), which resulted in toxic effects leading to reduced cell viability[4]. The neurotoxicity evolved to apoptotic cell death at 2.5 mM methylglyoxal. It was shown the intracellular mechanisms of such action through the MEK/Erk signaling pathway seemed to be upstream ROS generation and loss of cell viability. However, methylglyoxal in

concentrations unable to induce ROS generation (0.5 mM in cerebral cortex and 0.1 mM in hippocampus), provoked decreased cell survival, suggesting additional ROS-independent mechanisms related with methylglyoxal cytotoxicity during early brain development. These mechanisms could be associated, at least in part, with p38MAPK and JNK pathway activities. In addition, Toninello et al. [12] have shown a clearcut protection of rat liver mitochondria against the de-energizing action of either Ca^{2+}, or oxidizing agents (butylhydroperoxide and oxaloacetate). And Murata et al. [11] exhibit the antitumor effect of a polyamine biosynthetic pathway inhibitor methylglyloxalbis (butylamidinohydrazone) on human malignant melanoma cells. So, the usage of the low-doses ACS opens new therapeutics target in the anti-tumor therapy or states linked to ROS-dependent or independent mechanisms.

CONCLUSIONS

Thus, over the ultra-low impact of glyoxal (1.29×10^{-12} m) and methylglyoxal (1.04×10^{-12} M) cause effects in the different links of energy and nitrogen metabolism of lymphocytes *in vitro*, which is testified bysignificant changes in aldolase, LDH, AST, ALT activity, pyruvate concentration and glucose content in the culture medium. It should be noted that in the experimental conditions the nitrogen metabolism undergone less change, than energy. In most AST and ALT activity were changed in consequence of changes in intracellular energy exchange.

The results have revealed the activation of energy exchange of lymphocytes under ultra-weak influence of glyoxal and methylglyoxal, which was expressed in regular significant changes in the concentration of the main metabolites: pyruvate and glucose content in culture medium. This was a significant difference in glucose metabolism between experimental and control cultures of lymphocytes ($r= -0,67$, $p<0.05$), which was result of the super-weak impact glyoxal and methylglyoxal.

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Conflicts of Interest: The authors declare no conflict of interest.

Summary statement: It was shown for the first time that active carbonyl species in ultra-low doses could impact on the energy and nitrogen metabolism in lymphocytes cell culture.

REFERENCES

[1] Ellis EM Pharmacol Ther 2007;115(1):13–24.
[2] Turk Z Physiol Res. 2010;59:147–56.
[3] Kaushik DB BSP, Lingyun Wu BSP Recent Pat Cardiovasc Drug Discov 2007;2(2):89–99.
[4] Heimfarth L, Loureiro SO, Pierozan P, de Lima BO, Reis KP, Torres EB, et al Metab Brain Dis 2013;28(3):429–38.
[5] Zagayko AL, Voronina LN Krasilnikova OA Ukr Biopharm J 2010;5(10):52–7.
[6] Shangari N, Bruce WR, Poon R, O'Brien PJ Biochem Soc Trans 2003 ;31(6):1390–3.
[7] Amore A CR Nephrol Dial Transpl 2002;17(8):16–24.
[8] Lozinskyi LM SG Ukr Biochem J 2012;5(10):16–37.
[9] Akhand AA, Hossain K, Kato M, Miyata T, Du J, Suzuki H, et al Free Radic Biol Med 2001;31(10):1228–35.
[10] Bhattacharyya N, Pal A, Patra S, Haldar AK, Roy S, Ray M Int Immunopharmacol 2008;8(11):1503–12.
[11] Murata T, Hibasami H, Tagawa T, Nakashima K Anticancer Drugs 1992; 3(6):683-6.
[12] Toninello A, Siliprandi D, Castagnini P, Novello MC, Siliprandi N. Protective action of methylglyoxal bis (guanylhydrazone) on the mitochondrial membrane. Biochem Pharmacol. 1988 Sep 15;37(18):3395–9.
[13] Winchester RJ RG. Manual of Clinical Immunology. Rose NR FH, editor. Washington DC: American Society for Microbiology, 1976, pp. 64-76.
[14] Burlakova EB, Conradov AA, Maltseva EL Chem Phys 2003;22(2):21–40.
[15] Tietz NW et al. Clinical Guide to Laboratory Tests. 3rd ed. Philadelphia PA: WB Saunders Company, 1995. 374 p.
[16] Lapach SN, Chubenko AV BP. Statistical Methods in medical and biology research with excel exploitation. 2nd ed. Kyiv, MORYON, 2001. 367 p.

[17] Shangari N, Bruce WR, Poon R, O'Brien PJ Biochem Soc Trans 2003;31(6):1390–3.
[18] Liu G, Xu C, Feng L, Wang F Int J Mol Med 2015; 20:1019–27.
[19] Ottum MS, Mistry AM J Clin Biochem Nutr 2015;57(1):1–12.
[20] Luo X, Li R, Yan L-J J Diabetes Res 2015;2015:512-518.
[21] Sookoian S, Pirola CJ World J Gastroenterol 2015;21(213):711–25.