

Original Research Article

Peculiarities of Carbonyl Stress on Rats' Model of Diabetes and Rhabdomyolysis

ABSTRACT

Aim: the investigation of the carbonyl stress development on combined model of oxidative stress - glycerol stimulated rhabdomyolysis (RM) and hyperglycemia – streptozotocin induced diabetes (STZD) in rats.

Methodology: RM was induced by glycerol solution injection and proved by changes of heme oxygenase activity, creatine kinase activity, total heme, creatinine, uric acid, urea and bilirubin. STZD was induced by streptozotocin injection and proved by increasing of glucose levels. Carbonyl stress development was determined by total aldehyde levels, TBARS, protein CO group levels, CML and low-molecular-weight SH groups in blood plasma and liver tissue.

Results: It has been demonstrated significant increasing of carbonyl stress parameters under RM and STZD. But on combined model of RM and STZD carbonyl stress parameters were less than under STZD: total aldehyde levels were decreased in liver. Thus, that resulted in decreasing of TBARS and protein CO group levels. Low-molecular-weight SH groups were increased compare with STZD.

Conclusion: Changes of carbonyl stress parameters indicated the significant development of carbonyl stress under diabetes and rhabdomyolysis. It was demonstrated that additional stimulation of RM during hyperglycemia led to decreasing of carbonyl stress.

Keywords: carbonyl stress, oxidative stress, diabetes, rhabdomyolysis

1. INTRODUCTION

Carbonyl stress is the result of the transformation of lipid radicals formed during lipid peroxidation or the consequence of non-enzymatic protein glycosylation [1-2]. The most common reactive aldehydes found in biological systems are malondialdehyde, glyoxal, methylglyoxal, 3-deoxyglucosone, acrolein, etc. Lipid-derivatives aldehydes are more stable than their precursors free radicals and thus can diffuse from the initial site of lipid peroxidation and propagate oxidative injury by acting as toxic messenger [3]. These compounds are electrophilic, and thus are highly reactive with different cellular constituents majority of which are nucleophiles. Such strong nucleophilic sites as thiols, imidazole and hydroxyl groups of biomolecules as well as nitrogen and oxygen atoms in purine and pyrimidine bases are the most attractive targets for electrophilic attacks. Reactive endogenous aldehydes can bind covalently to Lys, His and Cys residues and form crosslinks in proteins that lead to disruption their structure and functions [4]. These changes resulted in irreversible molecules modifications and formation of variety adducts named advanced lipoxidation end products (ALEs) and advanced glycosylation end products (AGEs) (Fig. 1). There are pentosidine (cross-link interaction between Arg and Lys), carboxymethyllysine (oxidative degradation of the Amadori products, the reaction of glyoxal with Lys, and by

oxidation of both the Schiff base and the Amadori products), carboxyethyllysine (the reaction of methylglyoxal with and Lys), pyraline (the reaction of 3-deoxyglucosone with Lys) [5-6]. Protein carbonyl groups (CO) formation is the other protein modifications, which produced by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes (4-hydroxy-2-nonenal, malondialdehyde, acrolein) during lipid peroxidation [7-9]. The pathologic effects of AGEs/ALEs are related to their ability to promote oxidative stress and inflammation by binding with cell surface receptors, cross-linking with body proteins (such as collagen), altering their structure and function or modifying blood vessel structure [10-11]. The increasing in the steady-stage concentrations of AGEs and ALEs is related to hypertension, kidney and heart diseases, Alzheimer's and Parkinson's diseases, cataractogenesis, atherosclerosis, diabetes and diabetic secondary complications [12, 13]. The aim of the present study was the investigation of the carbonyl stress features on rats' experimental models of streptozotocin induced diabetes (STZD) and glycerol stimulated rhabdomyolysis (RM) and on combined model of these pathologies. RM, the dissolution of skeletal muscles, is characterized by the leakage of muscle-cell contents, including electrolytes, myoglobin, and other sarcoplasmic proteins into the blood circulation [14]. Myoglobin may release free ferrous (Fe^{2+}) ions, which could induce lipid peroxidation as a result of the generation of hydroxyl radicals via Fenton's reaction [15]. Diabetes is accompanied with chronic hyperglycemia, which leads to metabolism dysfunction and oxidative and carbonyl stress development [16-18].

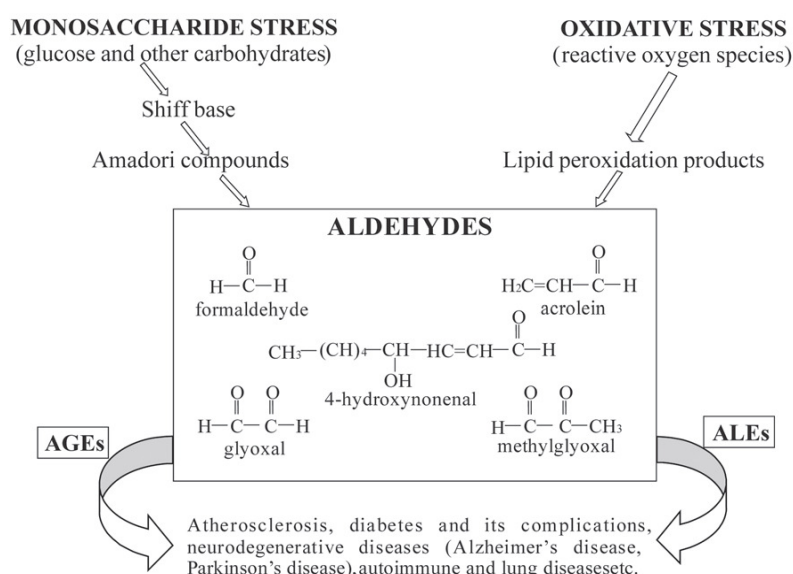


Fig.1. Formation of reactive aldehydes and advanced glycation and lipoxidation end products in oxidative and carbonyl stress development.

2. MATERIAL AND METHODS

2.1. Animals and experiment design

Experiments were performed on male Wistar rats weighed 180-220 g, which were maintained under diet consisted of concentrated granular food. Animals were randomly divided into 8 experimental groups (n=8 per group) as follows: group 1 control, groups 2-6 rhabdomyolysis (RM), group 7 diabetes (STZD), group 8 rhabdomyolysis + diabetes (RM + STZD).

For RM induction animals of 2-6 groups were injected with 50 % glycerol solution into both femoral muscles at dose 10 ml/kg. Animals were decapitated at 1st day (gr.2), 3rd day (gr.3), 4th day (gr.4), 6th day (gr.5), 10th day (gr.6) after glycerol injection.

Modeling of diabetes was performed by using the described technique [19]. For induction of STZD (Sigma, USA) the diabetic groups (gr.7, 8) were injected with streptozotocin (25 mg/kg in 0.5 ml of citrate buffer (pH 4.5)) in intraperitoneal injection three times: first and third time - at a dose of 25 mg/kg and second time - 20 mg/kg at weekly interval. 24 h before streptozotocin injection animals were given 1 ml of incomplete Freund's adjuvant in intraperitoneal injection.

Animals of group 8 were injected by glycerol solution at 10th day before decapitation.

2.2. Samples preparation

Samples of blood and liver were collected for investigation. The liver was homogenized in Tris-HCl buffer (pH 7.4) included 0.25 M saccharose. Blood and serum were separated by centrifugation at 3000 g for 10 min.

2.3. RM and STZD parameters determination

Under STZD blood was extracted from the tail vein for glucose analysis one week after last streptozotocin injection with glucometer "Glucofot Plus" (LLC "Diasvit", Ukraine).

Under RM heme oxygenase (HO) activity was determined in liver by the bilirubin formation [20]. Total heme content by pyridin hemochrome assay, creatine kinase activity by "Audit Diagnostics" kit (Ireland), bilirubin, creatinine, uric acid, urea by "Filicet Diagnostics" kits (Ukraine) were measured.

2.4. Carbonyl stress parameters determination

The concentration of aldehydes was determined by the purpald reagent (Sigma, USA) in liver using formaldehyde standard solutions [21].

Concentration of protein CO groups was quantified as described by Alamdari et al [22]. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). These modified CO groups were determined in plasma and liver by ELISA using rabbit anti-DNP Antibody (Sigma, USA). The absorbance was read spectrophotometrically at 490 nm using μ Quant (Biotek, USA).

The formation of MDA, an end product of fatty acid peroxidation was measured in liver spectrophotometrically at 532 nm (Biotek, USA) by using a thiobarbituric acid reactive substance (TBARS), essentially by the method of Genet et al [23]. 1,1,3,3-tetraethoxypropane was used as the standard.

Low-molecular-weight SH group levels were measured fluorimetrically by M.L. Hu using the fluorochrome o-phthalaldehyde (Fluka, Austria) in liver [24]. Excitation wavelength for o-phthalaldehyde was 360 nm and emission was detected at 420 nm by FL800 (Biotek, USA). Reduced glutation was used as standard (Sigma, USA).

N(6)-Carboxymethyllysine (CML) content in blood plasma was measured with using CML ELISA kit (Shanghai BlueGene Biotech CO., LTD, China).

2.5. Determination of protein concentration

Total protein concentrations were measured with biuret method, using bovine serum albumin as a standard.

2.6. Statistical analyses

The data are presented as mean±standard deviations (SD) for 7-8 animals in each group and analyzed using MS Excel 2007. Comparisons were made within each group and between groups using t-test for significant differences. P value <0.05 was considered as significant.

3. RESULTS AND DISCUSSION

3.1. Glycerol stimulated rhabdomyolysis development

It was shown that at the 1st day after glycerol injection total content of free heme increased by 10 times as result of hemoproteins, such as myoglobin releasing from damaged cells. From 3rd to 10th day of experiment this parameter decreased but at 10 day it still higher than control. The fast increasing of creatine kinase activity in blood plasma indicated the damage of muscle cells. At the 1st day of experiment this parameter was more than 10-fold higher than control, and then was gradually decreased at the same way as heme. Correlation coefficient between these parameters was 0.99 that indicated about muscles degradation and intracellular components releasing in blood. One of the organism protective mechanisms from toxic effects of heme is heme utilization in HO reaction. At 1st – 3rd day after glycerol injection HO activity levels was 5-6 times higher compared with control that indicated about induction of this enzyme. As result of HO functioning heme group transforms to bilirubin that led to fast increase of it in blood. At 1st – 3rd day of experiment bilirubin levels were more than 7-fold higher compared with control. Although this parameter is declining gradually, at 10th day of experiment it was 4 times higher than control. The increase of creatinine, urea and uric acid in plasma characterized the impairment of excretory function of kidneys. Changes of these parameters proved the RM development after glycerol injection (Table 1).

Table 1. Biochemical parameters in the blood plasma and liver of Wistar rats under rhabdomyolysis (mean±standard deviation, n=8).

Parameters	1 control	2 1 st day	3 3 rd day	4 6 th day	5 10 th day
Blood plasma					
Total heme, µmol/l	11±5	120±26*	45±9*	41±10*	34±9*
Creatine kinase activity, µmol/min	32±10	350±95*	184±77*	68±19*	53±18*
Creatinine, µmol/l	62±9	95±9*	79±10	130±18*	243±27*
Uric acid, mmol/l	0.11±0,04	0.19±0,05	0.22±0,05*	0.28±0,07*	0.39±0,11*
Urea, mmol/l	2.4±0.3	4.0±0.5*	6.5±1.0*	8.7±1.6*	8.5±1.7*
Bilirubin, µmol	4.6±0.6	31±4*	34±6*	20±4*	18±4*
liver					
Heme oxygenase activity, nmol of bilirubin/min/mg of protein	8,1±2,0	47±7*	51±8*	38±5*	22±7*

*p<0.05 compared with the control.

3.2. Streptozotocin induced diabetes development

For STZD proving glucose blood levels were measured one week after last streptozotocin injection. Plasma glucose concentration was 27 ± 8 mmol/l (groups 7 and 8) compared with control 6.5 ± 1.7 mmol/l.

3.3. Changes of carbonyl stress parameters under RM and STZD.

Under RM and STZD development carbonyl stress development was revealed by significant increasing of carbonyl stress parameters. At 4th day after glycerol injection total aldehyde level was increased by 3.3 times in liver under STZD and by 3 times under RM compared with control (Fig.2. (A), all results were equal to 1). This result indicated about reactive carbonyl compounds formation that led to modifications of biological molecules.

The TBARS levels were increased in liver under RM by 1.6 times, under STZD by 4.2 times in liver and by 4 times in plasma (is not presented) compared with control. These changes indicated about intensification of lipid peroxidation. MDA, the major lipid peroxidation product, can react with the free amino group of proteins, phospholipids and nucleic acids leading to their structural modification [25]. The TBARS levels under STZD were 2.6 times higher than under RM that indicated about the predominance of lipid peroxidation in STZD (Fig.2. (B)).

The attack of ROS against proteins modifies amino acids; lysine, arginine, proline and histidine residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as parameter of protein damage. Thus, the increasing of protein CO group levels by 3.4 times under RM and by 5 times under STZD in plasma compared with control was observed. It also showed that protein CO group level under STZD is significantly higher than under RM. In liver protein CO group levels were increased under RM by 3 times and under STZD by 3.9 times compared with control. It was also demonstrated the higher level of protein carbonylation under STZD than under RM. These changes can be considered as carbonyl stress consequences. The results are presented at Fig.2 (C).

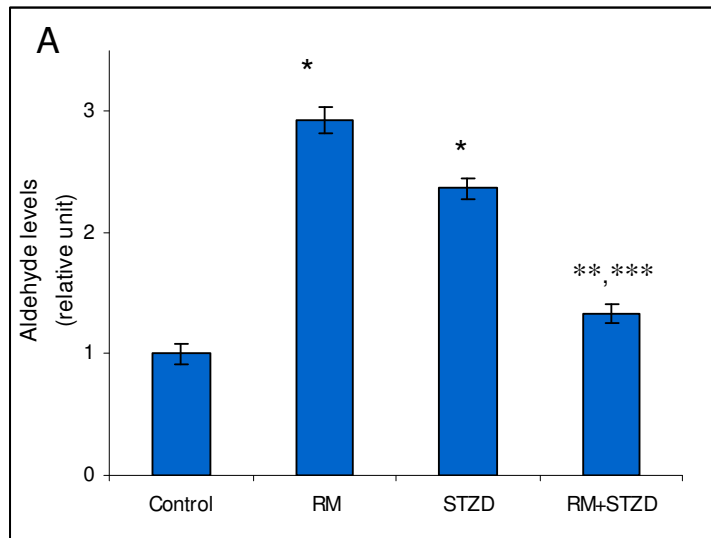
Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation. Carbonyl stress development led to accumulation of protein modifications such as AGEs. CML is an advanced glycation end product formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) reactions. CML as result of glyoxal and lysine interaction is known to accumulate in proteins with age and at an accelerated rate in diabetes [26]. It was shown that CML level was increased by 3 times in blood plasma (0.90 ± 0.03) mg/l compared with control (0.30 ± 0.05) mg/l under STZD. It was shown decreasing of low-molecular-weight SH group level in liver (3.6 ± 0.3) nmol/mg of protein by 2.2 times compared with control (8.0 ± 0.6) nmol/mg of protein under STZD. SH groups take place in protecting molecules against electrophiles and free radicals. This is due to their nucleophilicity and to the high reaction rate of thiols with free radicals.

3.4. Changes of carbonyl stress parameters on combined model of RM and STZD.

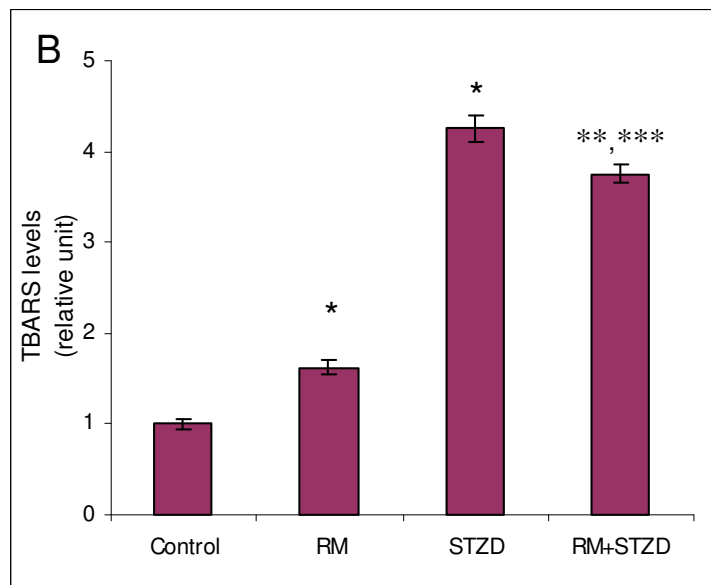
The high level of aldehydes and the increasing of carbonyl stress parameters proved the carbonyl stress development under RM and STZD. Thus, it was suggested that under mutual development of RM and STZD it will be an intensive increasing of carbonyl stress development. But obtained results were different from expected.

Under simultaneous development of RM and STZD, the total aldehyde level in liver was decreased by 2.6 times compared with STZD and by 2.4 times compared with RM. That resulted in decreasing of TBARS by 2.3 times compared with RM and not significant changes for STZD. It was also observed the decreasing of protein CO group levels by 4.2

204 times in plasma and by 3.6 times in liver compared with STZD, and by 2.9 times in blood
 205 plasma and by 2.8 times in liver compared with RM. CML level was not significant changed
 206 in plasma (0.80 ± 0.06) mg/l compared with STZD. Low-molecular-weight SH groups were
 207 increased by 1.3 times in liver (4.5 ± 0.4) nmol/mg of protein compared with STZD.
 208



209
 210



211
 212

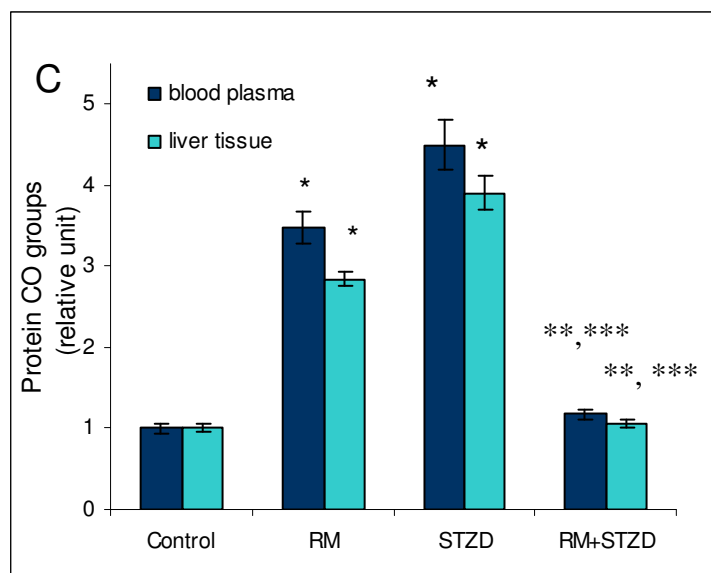


Fig.2. Changes of carbonyl stress parameters under rhabdomyolysis (RM) and diabetes (STZD): total aldehyde levels in liver (A), thiobarbituric acid reactive substances (TBARS) in liver (B) and levels of protein CO groups in blood plasma and in liver (C), all results were equal to 1. * $p < 0.05$ compared with the control, ** $p < 0.05$ compared with the RM, * $p < 0.05$ compared with the STZD.**

4. CONCLUSION

The present study showed that changes of main carbonyl stress parameters such as total aldehyde level, TBARS, protein CO group level, low-molecular-weight SH group level and concentration of CML in blood and plasma of rats indicated the intensification of carbonyl stress under RM and STZD development. It was shown that additional stimulation of RM in animals under STZD led to significant decreasing of carbonyl stress intensity compared with STZD.

ETHICAL APPROVAL

All *in vivo* experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals, used for experimental and other scientific purposes" (Strasburg, 1985).

REFERENCES

1. Niki E, Yoshida Y, Saito Y, Noguchi N. Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem Biophys Res Commun.* 2005; 338 (1): 668-676. PubMed PMID: 16126168
2. Tessier FJ. The Maillard reaction in the human body. The main discoveries and factors that affect glycation. *Pathol Biol.* 2010; 58(3): 214-219. PubMed PMID: 19896783
3. Liu XY, Zhu M.X, Xie JP. Mutagenicity of acrolein and acrolein-induced DNA adducts. *Toxicol Mech Meth.* 2010; 20 (1): 36-44. PubMed PMID: 20158384

- 247 4. Fritz KS, Petersen DR. Exploring the biology of lipid peroxidation-derived protein
248 carbonylation. *Chem Res Toxicol.* 2011; 24 (9): 1411-1419. PubMed PMID:
249 21812433
- 250 5. Pamplona R. Advanced lipoxidation end-products. *Chem Biol Interact.* 2011; 192 (1-
251 2): 14-20. PubMed PMID: 21238437
- 252 6. Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects
253 for diabetic complications. *JAMA.* 2002; 288 (20): 2579-2588. PubMed PMID:
254 12444865
- 255 7. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups
256 as biomarkers of oxidative stress. *Clin Chim Acta.* 2003; 329 (1-2): 23-38. PubMed
257 PMID: 12589963
- 258 8. Uchida K. Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol*
259 *Med.* 2000; 28 (12): 1685– 1696. PubMed PMID: 10946210
- 260 9. Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and
261 disease. *Chem Res Toxicol.* 1997; 10 (5): 485– 494. PubMed PMID: 9168245
- 262 10. Vlassara H. The AGE-receptor in the pathogenesis of diabetic complications.
263 *Diabetes Metab Res Rev.* 2001; 17 (6): 436–443. PubMed PMID: 11757079
- 264 11. Inagaki Y, Yamagishi S, Okamoto T, Takeuchi M, Amano S. Pigment epithelium-
265 derived factor prevents advanced glycation end products-induced monocyte
266 chemoattractant protein-1 production in microvascular endothelial cells by
267 suppressing intracellular reactive oxygen species generation. *Diabetologia.* 2003; 46
268 (2): 284-287.
- 269 12. Jay D, Hitomi H, Griendling K. Oxidative stress and diabetic cardiovascular
270 complications. *Free Radic Biol Med.* 2006; 40 (2): 183-192. PubMed PMID:
271 16413400
- 272 13. Yamagishi Y. Role of advanced glycation end products (AGEs) and receptor for
273 AGEs (RAGE) in vascular damage in diabetes. *Exp Gerontol.* 2011; 46 (4): 217-224.
274 PubMed PMID: 21111800
- 275 14. Bosch X, Poch E, Grau JM. Rhabdomyolysis and acute kidney injury. *N Engl J Med.*
276 2009; 361 (1): 62-72. PubMed PMID: 19571284
- 277 15. Rice-Evans C, Green E, Paganga G, Cooper C, Wrigglesworth J. Oxidised low
278 density lipoproteins induce iron release from activated myoglobin. *FEBS Lett.* 1993;
279 326 (1-3): 177–182. PubMed PMID: 8391992
- 280 16. Rebolledo OR, Actis Dato SM. Postprandial hyperglycemia and hyperlipidemia-
281 generated glycoxidative stress: its contribution to the pathogenesis of diabetes
282 complications. *Eur Rev Med Pharmacol Sci.* 2005; 9 (4): 191–208. PubMed PMID:
283 16128039
- 284 17. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis
285 of diabetic neuropathy. *Endocr Rev.* 2004; 25(4): 612–628. PubMed PMID:
286 15294884
- 287 18. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism.
288 *Diabetes.* 2005; 54 (6): 1615-1625.
- 289 19. Zakir'janov AR, Velikij DA, Onishchenko NA, Klimenko ED, Pozdnjakov OM,
290 inventors; Method of modeling type I diabetes mellitus in rats. Russian Federation
291 patent RU 2 400 822, ICP G09B N23/28. 2010 Sept 27. (Russian)
- 292 20. Sardana MK, Sassa S, Kappas A. Hormonal regulation of heme oxygenase
293 induction in avian hepatocyte culture. *Biochem Pharmacol.* 1985; 34 (16): 2937-
294 2944. PubMed PMID: 2992523
- 295 21. Lee CH, Tsai CM. Quantification of bacterial lipopolysaccharides by the purpald
296 assay: Measuring formaldehyde generated from 2-keto-3-deoxyoctonate and
297 heptose at the inner core by periodate oxidation. *Anal Biochem.* 1999; 267 (1): 161–
298 168. Pubmed PMID: 9918668

- 299 22. Alamdari DH, Kostidou E, Paletas K. et al. High sensitivity enzyme-linked
300 immunosorbent assay (ELISA) method for measuring protein carbonyl in samples
301 with low amounts of protein. Free Radic Biol Med. 2005; 39 (10): 1362–1367.
302 PubMed PMID: 16257645
- 303 23. Genet S, Kale RK, Baquer NZ. Alterations in antioxidantenzymes and oxidative
304 damage in experimental diabetic rat tissues: effect of vanadate and fenugreek
305 (Trigonellafoenum graecum). Mol Cell Biochem. 2002; 236 (1-2): 7–12. PubMed
306 PMID: 12190123
- 307 24. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Methods
308 Enzymol. 1994; 233: 380–385. PubMed PMID: 8015473
- 309 25. Kingsley M, Cunningham D, Mason L, Kilduff LP, McEneny J. Role of creatine
310 supplementation on exercise-induced cardiovascular function and oxidative stress. J
311 Oxid Med Cell Longev. 2009; 2(4): 247-254. Pubmed PMID: 20716911
- 312 26. Baynes JW. Role of oxidative stress in development of complications in diabetes.
313 Diabetes. 1991; 40 (4): 405-412. Pubmed PMID: 2010041
- 314
- 315
- 316