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THE INTENSITY OF FREE RADICAL PROCESSES IN THE LIVER MITOCHONDRIAL FRACTION UNDER THE CONDITIONS OF TOXIC INJURY AND CORRECTION BY THE 3,4-DIHYDROPYRIMIDINE-2-ONE AMMONIUM DERIVATIVE

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The research deals with the determination of the superoxide-anion radical, TBA-active products and protein carbonyl derivatives content in the protein-deficient rat liver mitochondrial fraction under the conditions of acetaminophen-induced hepatitis and correction by the 3,4-dihydropyrimidine-2-one ammonium derivative. The researches were conducted on white rats of 90-100 g body mass aged 2-2.5 months. There were used rats, which according to the experimental model were separated into the following groups: I – animals receiving full-value semi-synthetic ration (C); II – animals receiving low-protein ration (LPR); III – animals with acetaminophen-induced liver injury receiving complete ration (H); IV – animals with acetaminophen-induced liver injury that were previously maintained on semi-synthetic low-protein ration (LPR+H); V-XII – animals, which were introduced with the examined 3,4-dihydropyrimidine-2-one ammonium derivative at concentrations of 5; 10; 15; 20; 25; 30; 40; 50 mg/kg of the body weight correspondingly. The examined compound concentrations were introduced orally in 2 hours after acetaminophen exposure during 2 days according to GLP recommendation. The most pronounced antioxidant effect at concentration of 15 mg/kg of the 3,4-dihydropyrimidine-2-one ammonium derivative was established. It is observed that the introducing of the examined compound in the specified concentration leads to the maximal decrease of superoxide-anion radical, TBA-active products and protein carbonyl derivatives content in the rat liver mitochondrial fraction with toxic injury to control values. The established patterns are confirmed by a correlation analysis, which was shown the existence of a close correlation between the concentration of test compound and superoxide-anion radical content. The 3,4-dihydropyrimidine-2-one ammonium derivative is a promising compound for the creation on its basis of an antioxidant agent which is capable to effectively suppression of the intensity of free radical processes in the liver mitochondria under the conditions of toxic injury.

Key words: the 3,4-dihydropyrimidine-2-one ammonium derivative, superoxide-anion radical, TBA-active products, protein carbonyl derivatives, mitochondrion

Introduction. One of the reasons for the development of various pathological conditions underlying many diseases of the liver, including toxic lesions, is the intensification of free radical processes with the enhanced formation of reactive oxygen metabolites (Li et al., 2015; Galimova, 2012). Since the liver performs the most important metabolic function in the body, its cells are the primarily subject to oxidative damage due to the reactive oxygen species (ROS), which is accompanied by an oxidative modification of the main macromolecules (Whaley-Connell et al., 2011; Rahman et al., 2012). As a result, there is an imbalance in the mechanisms of regulation of cellular homeostasis, there is a violation of the functioning of metabolic pathways, enzymatic systems, transport systems, which leads to a shortage of energy supply in liver cells. In this regard, the development of oxidative stress inevitably causes a violation of the liver functional state (Jadeja et al., 2017; Muriel and Gordillo, 2016).

Today, clinical practice has introduced a variety of antioxidant and hepatoprotective drugs, whose action is aimed to preventing the development of oxidative stress in the liver cells and the retrieval of antioxidant status of organism (Mishra et al., 2014). The pharmaceutical market for modern antioxidants is represented by both natural compounds and synthetic preparates characterized by a number of side effects and contraindications, or unstable with prolonged storage. At the same time, the application of antioxidants is limited by the complexity of selecting their effective concentrations, the lack of clarity of the laws of metabolic transformations of drugs in the body, various restrictions in the use, etc. (Yoshihara et al., 2010; Nimse and Pal, 2015). Therefore, the search for substances-antioxidants, which suppresses the intensity of free radical processes in liver cells, remains relevant.

In the context of solving this problem it can be promising derivatives of ammonium salts of 3,4-dihydropyrimidine-2-onone, which due to its structural analogy with clinically active dihydropyrimidines, can potentially have a wide spectrum of pharmacological activity (Harika et al., 2014). In this case, the biological action of

derivatives of dihydropyrimidines largely depends on the nature and degree of their functionalization.

Therefore, the aim of the current work was to research the intensity of free radical processes in the liver mitochondrial fraction of protein-deficient rats under the conditions of toxic injury and correction by the 3,4-dihydropyrimidine-2-one ammonium derivative.

Materials and Methods. The experiments were conducted on white rats of 90-100 g body mass aged 2-2.5 months. The experiment was conducted in accordance with the rules set by the 'European convention for the protection of vertebrate animals used for experimental and other scientific purposes' (Strasbourg, 1986).

The animals were separated into solitary plastic cages with sand bedding and *ad libitum* access to water.

The daily rations were regulated according to principles of pair feeding. The animals were separated into the following experimental groups:

I – animals receiving full-value semi-synthetic ration (C);

II – animals receiving low-protein ration (LPR);

III – animals with acetaminophen-induced liver injury receiving complete ration (H);

IV – animals with acetaminophen-induced liver injury that were previously maintained on semi-synthetic low-protein ration (LPR+H).

The animals of the groups I and III received a standard ration containing 14% of protein (casein), 10% of fat, and 76% of carbohydrates, balanced by all the essential nutrients. The animals of the groups II and IV received isoenergetic ration containing 4.7% of protein, 10% of fat, and 85.3% of carbohydrates, calculated after recommendations of the American Institute of Nutrition (Kuvandik et al., 2008).

The animals were maintained on the corresponding diet during four weeks. Afterwards, the acetaminophen-induced liver injury was modeled by *per os* administration of 2% starch suspension of acetaminophen in daily dose 1250 mg/kg (0,5 LD_{50}) of the body weight during 2 days.

V-XII – animals, which were introduced with 3,4-dihydropyrimidine-2-one ammonium derivative at concentrations of 5; 10; 15; 20; 25; 30; 40; 50 mg/kg of the body weight correspondingly.

The examined compound concentrations were introduced by *per os* administration in 2 hours after acetaminophen exposure during 2 days according to GLP (Good Laboratory Practise) recommendation.

The examined 3,4-dihydropyrimidine-2-one ammonium derivative is kindly provided by the Departament of Organic and Physical Chemistry and Ecology of Chemical Production from the Institute of Biology, Chemistry and Natural Resources, Fedkovych Chernivtsi National University.

Cervical dislocation was performed under the light ether anesthesia on day 31 of the experiment.

Mitochondrial fraction was separated by differential centrifugation in the following buffer medium: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) at 0-3 °C.

The rate of superoxide anion radical generation in mitochondrial fraction was measured with nitro blue tetrazolium test (NBT) (Kopylchuk and Voloshchuk, 2016).

The concentration of TBA-active products was assessed by the reaction with 2-thiobarbituric acid (TBA), occurring at high temperature in acidic environment, and forming the colored complex, determined at λ 532 nm ($\varepsilon = 1.56\times10^5$ $M^{-1}\times cm^{-1}$). The concentration of TBA-active products was expressed in nmol/mg of protein (Andreeva et al., 1988).

Protein carbonylation was assessed via amount of 2.4-dinitrophenylhydrazone derivatives, produced in reactions of oxidized amino acid residues with 2.4-dinitrophenylhydrazine, and expressed as nmol of carbonyl protein derivatives per mg of protein (Zaytseva and Shandrenko, 2012).

The protein content was determined according to the Lowry method.

The correlation between the concentration of the test compound and the intensity of superoxide-anion radical generation was established by the Pirson correlation coefficient (Prozorovskiy, 2007).

The data statistics was processed with MS Excel software, and represented as mean ± deviation. The statistical significance was determined with standard Student's *t*-test.

Results and discussion. We examined the antioxidant effect of 3,4-dihydropyrimidine-2-one ammonium salts derivatives in concentrations of 5; 10; 15; 20; 25; 30; 40; 50 mg/kg in the *in vivo* system. A significant decrease of the superoxide-anion radical content to the control values is observed in the mitochondrial fraction of rats with toxic hepatitis under the conditions of the compound introduction at a dose of 15 and 20 mg / kg (fig. 1). In this case, introduction of the examined compound at a dose of 15 mg/kg demonstrates the most pronounced superoxide-inhibiting effect.

The establishing correlation relations between the concentration of 3,4-dihydropyrimidine-2-one ammonium salts derivative and the content of anion-radical superoxide showed the existence of a close correlation between the examined parameters (fig. 2).

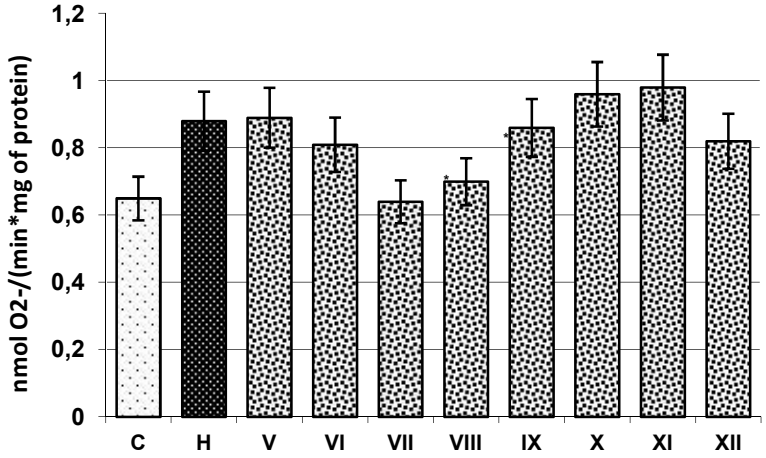


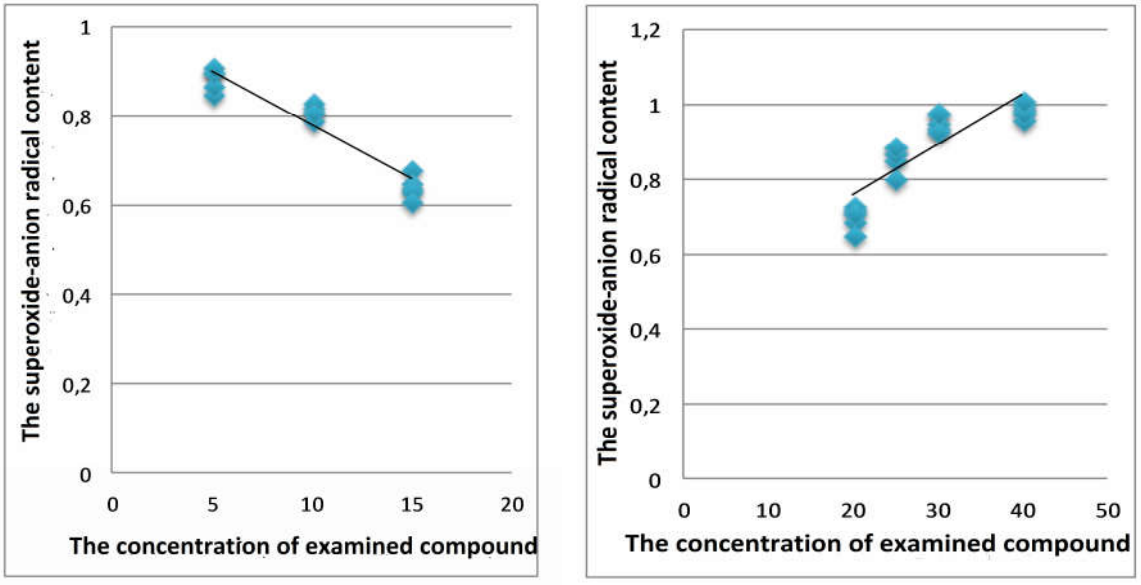
Fig. 1. Superoxide-anion radical content in the rat liver mitochondrial fraction under the conditions of toxic injury and administration of different concentrations of 3,4-dihydropyrimidine-2-one ammonium derivative

*Note: *Significantly different from the group of animals with toxic liver injury receiving complete ration (H), P≤0,05; V-XII – animals, which were introduced with 3,4-dihydropyrimidine-2-one ammonium derivative in concentrations of 5; 10; 15; 20; 25; 30; 40; 50 mg/kg of the body weight accordingly.*

The decrease of the superoxide-anion radical content was established by the introduction of examined compound at a dose of 5 to 15 mg/kg (fig. 2) with maximum suppression of superoxide generation at concentration of 15 mg/kg. At the indicated range of the examined compound concentrations, the correlation coefficient becomes to value -0.96, which indicates a close inverse relationship between the examined compound concentration and the content of superoxide. At the same time, with an increase of the examined compound concentration (at the range of doses of 20-50 mg/kg), there is a direct proportional relationship between the compound concentration and the content of superoxide (fig. 2), which indicates the prooxidant effect of high doses of the compound.

At the next stage of our work, we examined the effect of 3,4-dihydropyrimidine-2-one ammonium salts derivative at concentration of 15 mg/kg on the intensity of free radical processes under the conditions of toxic liver injury on the background of alimentary protein deficiency.

The most pronounced intensification of free radical processes is observed in the liver mitochondria of protein-deficient rats with toxic injury (fig. 3.). In this case, the introduction of examined compound at concentration of 15 mg/kg leads to the retrieval of the intensity of superoxide generation to the control values.



5-15 mg/kg ***20-50 mg/kg***

Fig. 2. The correlation between the concentration of examined compound (x) and the superoxide-anion radical content (y)

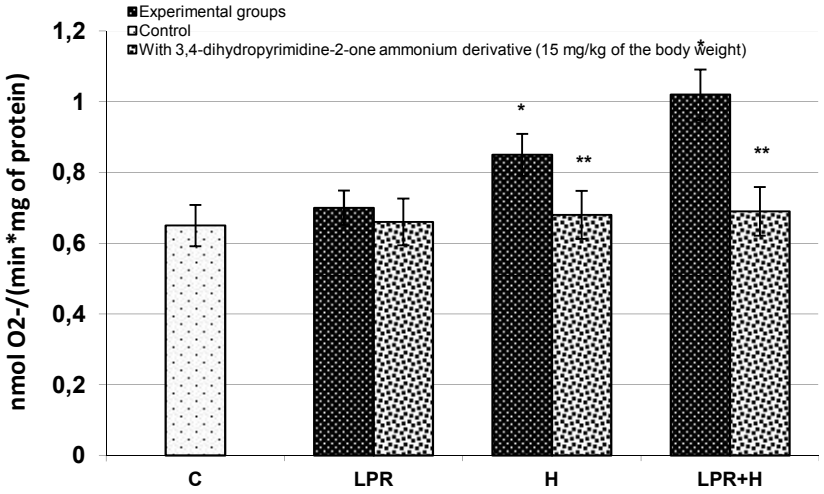


Fig. 3. Superoxide-anion radical content in the rat liver mitochondrial fraction under the conditions of acetaminophen-induced hepatitis on the background of alimentary protein deprivation and correction by the 3,4-dihydropyrimidine-2-one ammonium derivative

*Note (here and forwards): *Significantly different from the control (C), P≤0,05*

*** - Significantly different from the group of animals which did not receive the examined compound, P≤0,05*

It is known that superoxide-anion radical is formed as a result of "leakage" of electrons transported along the respiratory chain. The main place of generation of reactive oxygen species in mitochondria are the I and III complexes of the respiratory chain. Since mitochondria concentrate most of the oxidative metabolic pathways and contain numerous redox carriers and centers that are capable of producing active forms of oxygen, under the conditions of the increase of superoxide generation, which was shown by us, it is likely that biomolecules primarily of mitochondria will be damaged (Bhatacharya, 2015; Circu and Aw, 2010).

In particular, complexes of the electron transport chain (iron-sulfur clusters of complex I), mitochondrial DNA (hydroxyl groups of deoxyribose, amine groups of nitrogenous bases), and lipids of the outer and inner mitochondria membrane are primarily sensitive to ROS action. Among the lipids, the target for ROS action are also unsaturated fatty acids, which include arachidonic, linoleic and docosahexaenoic acids, whose damage initiates the process of peroxide lipid oxidation. As a result, secondary products of lipid modification are formed, including TBA-active products (Pizzimenti et al., 2013).

The most pronounced increase of TBA-active products content is observed in rats with acetaminophen-induced liver injury on the background of alimentary protein deficiency (fig. 4.). At the same time, the introduction of examined compound at concentration of 15 mg/kg leads to a decrease of TBA-active products content to the control values.

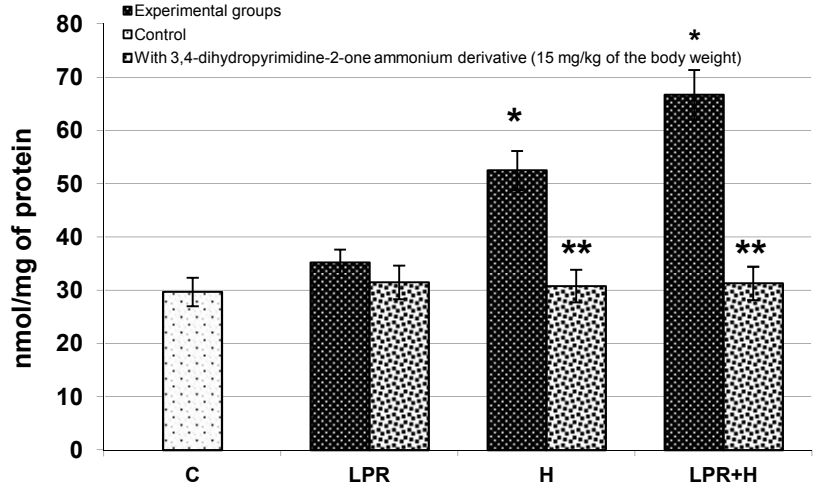


Fig. 4. TBA-active products content in the rat liver mitochondrial fraction under the conditions of acetaminophen-induced hepatitis on the background of alimentary protein deprivation and correction by the 3,4-dihydropyrimidine-2-one ammonium derivative

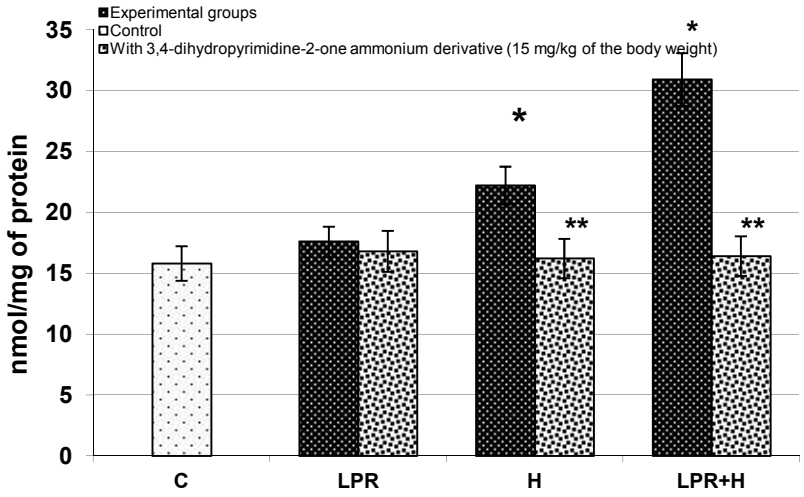


Fig. 5. Protein carbonyl derivatives content in the rat liver mitochondrial fraction under the conditions of acetaminophen-induced hepatitis on the background of alimentary protein deprivation and correction by the 3,4-dihydropyrimidine-2-one ammonium derivative

In addition, the sensitive targets for the action of reactive oxygen species are mitochondrial proteins (Barelli et al., 2008). Due to their oxidation modifications, stable carbonyl derivatives can be formed. The most commonly amino acids subjected to oxidative modifications are proline, arginine, lysine and threonine, with the corresponding carbonyl derivatives being formed: 2-pyrrolidone, glutamyl semialdehyde, aminoadipisemialdehyde and 2-amino-3-ketobutyryl acid (Ayala et al., 2014).

The maximum accumulation of protein carbonyl derivatives was established in the liver mitochondrial fraction of protein-deficient rats with toxic injury (fig. 5.). At the same time, the introduction of examined compound at concentration of 15 mg/kg leads to a decrease of protein oxidative modification products content to the control values.

Conclusions. Thus, the established results allow to make a conclusion that the 3,4-dihydropyrimidine-2-one ammonium derivative is a promising compound for the creation on its basis of an antioxidant agent which is capable to effectively suppression of the intensity of free radical processes in the liver mitochondria under the conditions of toxic injury.

References

1. Li S., Tan H., Wang N. The role of oxidative stress and antioxidants in liver diseases // International Journal of Molecular Sciences. – 2015. – Vol. 16. – P. 26087–26124. doi:10.3390/ijms161125942
2. Galimova S.F. Drug-induced liver injuries // The Russian Journal of Gastroenterology, Hepatology, Coloproctology. – 2012. – Vol. 3. – P. 38–48. (In Russian)
3. Whaley-Connell A., McCullough P.A., Sowers J.R. The role of oxidative stress in the metabolic syndrome // Reviews in Cardiovascular Medicine. – 2011. – Vol. 12. – P. 21–29. doi: 10.3909/ricm0555
4. Rahman T., Hosen I., Towhidul Islam M.M., Shekhar H.U. Oxidative stress and human health // Advances in Bioscience and Biotechnology. – 2012. – Vol. 3. – P. 997–1019. doi:10.4236/abb.2012.327123
5. Jadeja R.N., Devkar R.V., Nammi S. Oxidative stress in liver diseases: pathogenesis, prevention, and therapeutics // Oxidative Medicine and Cellular Longevity. – 2017. – Vol. 2017. – P. 834186–834189. doi: https://doi.org/10.1155/2017/8341286
6. Muriel P., Gordillo K.R. Role of oxidative stress in liver health and disease // Oxidative Medicine and Cellular Longevity. – 2016. – Vol. 2016. – P. 2. doi: http://dx.doi.org/10.1155/2016/9037051
7. Mishra S., Aeri V., Katare D.P. Hepatoprotective medications for liver injury // World Journal of Pharmacy and Pharmaceutical Sciences. – 2014. – Vol. 3 (5). – P. 891–932.
8. Yoshihara D., Fujiwara N., Suzuki K. Antioxidants: benefits and risks for long-term health // Maturitas Journal. – 2010. – Vol. 67, №2. – P. 103–107. doi: 10.1016/j.maturitas.2010.05.001
9. Nimse S.B., Pal D. Free radicals, natural antioxidants, and their reaction mechanisms // RSC Advances. – 2015. – Vol. 5. – P. 27986–28006. doi: 10.1039/C4RA13315C
10. Harika L.V., Kumar K.R., Shaik A.B. Synthesis, characterization and biological evaluation of 3,4-dihydropyrimidin-2(*1H*)-thione derivatives // Archives of Applied Science Research. – 2014. – Vol. 6, № 6. – P. 121-127.
11. Kuvandik G., Duru M., Nacar A., Yonden Z., Helvaci R., Koc A., Kozlu T., Kaya H., Sogüt S. Effects of Erdosteine on Acetaminophen-induced Hepatotoxicity in Rats // Toxicologic Pathology. – 2008. – 36(5). – P. 714–719. doi: 10.1177/0192623308320800
12. Kopylchuk G.P., Voloshchuk O.M. Pecularities of the free radical processes in rat liver mitochondria under toxic hepatitis on the background of alimentary protein deficiency // Ukr. Biochem. J. – 2016. – Vol. 88, № 2. – P. 66–72. doi: http://dx.doi.org/10.15407/ubj88.02.066

13. Andreeva L.I., Kozhemyakin L.A., Klishkun A.A. Modification of the method of lipid peroxides determination in the test with thiobarbituric acid // Laboratornoe delo. – 1988. – 11. – P. 41–44. (In Russian).
14. Zaytseva O.V., Shandrenko S.G. Modification of spectrophotometric method of determination of protein carbonyl groups // Ukr. Biochem. J. – 2012. – Vol. 84 (5). – P. 112–116.
15. Prozorovskiy V.B. Statistical processing of the pharmacological researches results // Psichofarmakologiya i biologicheskaya farmakologiya. – 2007. – Vol.7, № 3-4. – P. 2090–2120. (In Russian)
16. Bhatacharya S. Reactive oxygen species and cellular defense system // Free Radicals in Human Health and Disease. – 2015. – Vol. 2015. – P. 17–29. doi: 10.1007/978-81-322-2035-0_2
17. Circu M.L., Aw T.Y. Reactive oxygen species, cellular redox systems and apoptosis // Free Radical Biology and Medicine. – 2010. – Vol. 48, № 6. – P. 749-762. doi: 10.1016/j.freeradbiomed.2009.12.022
18. Pizzimenti S., Ciamporcero E., Daga M., Pettazzoni P., Arcaro A., Cetrangolo G., Minelli R., Dianzani C., Lepore A., Gentile F., Barrera G. Interaction of aldehydes derived from lipid peroxidation and membrane proteins // Front Physiol. – 2013. – Vol. 4. – P. 242–258. doi: 10.3389/fphys.2013.00242
19. Barelli S., Canellini G., Thadikkaran L., Crettaz D., Quadroni M., Rossier J.S., Tissot J.D., Lion N. Oxidation of proteins: basic principles and perspectives for blood proteomics // Proteomics Clinical Applications. – 2008. – Vol. 2. – P. 142–157. doi: 10.1002/prca.200780009
20. Ayala A., Munoz M.F., Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal // Oxidative Medicine and Cellular Longevity. – 2014. – Vol. 2014. – P. 1–31. doi: http://dx.doi.org/10.1155/2014/360438

ІНТЕНСИВНІСТЬ ВІЛЬНОРАДИКАЛЬНИХ ПРОЦЕСІВ У МІТОХОНДРІАЛЬНІЙ ФРАКЦІЇ ПЕЧІНКИ ЗА УМОВ ТОКСИЧНОГО УРАЖЕННЯ ТА КОРЕКЦІЇ АМОНІЄВИМ ПОХІДНИМ 3,4-ДИГІДРОПІРИМІДИН-2-ОНУ

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У роботі визначено вміст супероксид-аніон радикалу, ТБК-активних продуктів та білкових карбонільних похідних у мітохондріальній фракції печінки білок-дефіцитних щурів за умов ацетамінофен-індукованого ураження та корекції похідним амонієвих солей 3,4-дигідропіримідин-2-ону. Дослідження проведено на білих безпородних щурах масою 90 – 100 г, віком 2 – 2,5 місяці. У експерименті було використано щурів, яких згідно з моделлю дослідження розділили на такі групи: I група – щурі, які перебували на повноцінному напівсинтетичному раціоні (К); II група – щурі, які перебували на напівсинтетичній низькопротеїновій дієті протягом 28 днів (1/3 добової потреби білка) (НПР); III – щурі з токсичним ураженням печінки, які перебували на повноцінному раціоні (ТУ); IV група – щурі, яким моделювали гостре ацетамінофен-індуковане токсичне ураження печінки на фоні аліментарної депривації протеїну (НПР+ТУ); V-XII група – щурі, яким вводили досліджуване похідне амонієвих солей 3,4-дигідропіримідин-2-ону у концентраціях 5; 10; 15; 20; 25; 30; 40; 50 мг/кг маси тварини відповідно. Досліджувані концентрації сполук вводили шляхом per os за допомогою спеціального зонду через 2 год після введення ацетамінофену протягом двох діб згідно рекомендації GLP. Встановлено, що найбільш виражений антиоксидантний ефект проявляє амонієве похідне 3,4-дигідропіримідин-2-ону в концентрації 15 мг/кг. Показано, що введення досліджуваної сполуки у вказаній концентрації призводить до максимального зниження показників вмісту супероксид-аніон радикалу, ТБК-активних продуктів та карбонільних дериватів у мітохондріальній фракції печінки щурів із токсичним ураженням до показників контролю. Встановлені закономірності підтверджуються кореляційним аналізом, що показав існування тісного кореляційного зв'язку між концентрацією досліджуваної сполуки та вмістом супероксид-аніон радикалу. Зроблено висновок, що похідне амонієвих солей 3,4-дигідропіримідин-2-ону є перспективною сполукою для створення на її основі антиоксидантного засобу, здатного ефективно інгібувати інтенсивність вільнорадикальних процесів у мітохондріях печінки за умов її токсичного ураження.

Ключові слова: амонієві похідні 3,4-дигідропіримідин-2-ону, супероксид-аніон радикал, ТБК-активні продукти, карбонільні похідні протеїнів, мітохондрії

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