Changes of 8-Oxo-2'-Deoxyguanosine Level in Mouse Liver Cells DNA in Case of Acute Toxic Stress*

N.V. Marmiy@, D.S. Esipov

Faculty of Biology, Lomonosov Moscow State University, Moscow, 119991 Russia
@Corresponding author e-mail: marmiynv@gmail.com

The changes of the 8-oxo-2'-deoxyguanosine (8-oxo-dG)/dG ratio in the DNA of laboratory mice hepatocytes under the influence of toxic stress were studied. It was shown that the injection of carbon tetrachloride causes the growth of 8-oxo-dG level. A rapid increase in the level of 8-oxo-dG in DNA occurs during the first day of the experiment at short-term toxic stress. Subsequently, 48 hours after the initial injection, the level of 8-oxo-dG decreases to the control values. This change in the value of the biomarker can be attributed to the activation of the reparative and antioxidant systems. The subsequent injection results again in an increase of 8-oxo-dG level, and the latter only increases thereafter. This reflects the exhaustion of the reparative potential of the organism and accompanies the progress of inflammation and pathological change of the liver tissue.

Keywords: *8-oxo-2'-deoxyguanosine, induced cirrhosis, biomarker of oxidative stress, reversed phase HPLC, electrochemical detection.*

Introduction

8-Oxo-2'-deoxyguanosine is a commonly accepted biomarker of oxidizing stress and related diseases [1]. As a rule, its content in cellular DNA increases in case of pathologies and after stress and reverts to the normal amounts due to adequate therapy or elimination of the stress factor. The latter is due to the fact that the cells of all living organisms have a reparative mechanism of 8-oxo-dG removal from the DNA chain followed by its replacement with normal 2'-deoxyguanosine. Thanks to such rather rapid changes of this compound content in DNA in response to external and internal factors it can be used as an "early" biomarker of the efficiency of drug therapy and other types of therapy. In particular, there are studies that revealed correlations between the change of 8-oxo-dG level in the blood and urine of cancer patients subjected to chemotherapy and their subsequent survival rate [2]. Besides, according to literature data 8-oxo-dG is used for a long time as a biomarker of autoimmune [3], inflammatory [4], allergic [5] and neurodegenerative diseases [6], as well as aging [7] and response to stress [8].

* *Original Russian Text © N.V. Marmiy, D.S. Esipov, 2016, published in Tonkie Khimicheskie Tekhnologii /Fine Chemical Technologies, 2016, Vol. 11, No. 6, pp. 68–74.*

Besides, in recent years there are data in literature that 8-oxo-dG can act as a regulatory molecule affecting the activity of a number of antioxidative and reparative complexes and also the process of inflammatory reactions [9–13]. In particular, exogenous 8-oxo-dG can inhibit the RAC/STAT cascade and the related functions, such as the synthesis of inflammatory cytokines and prostaglandins, phagocytosis and the production of active forms of oxygen by immunocompetent cells [9, 10]. Due to this 8-oxo-dG can exert anti-inflammatory [11, 12] and antiallergic [13, 14] action. According to the experimental data of the Korean research group, 8-oxo-dG applied in high doses reduced the intensity of symptoms of bronchial asthma [13], autoimmune encephalomyelitis [12], stomach ulcer [11] and ovalbumin allergy [14] in experimental animals. Besides, this compound considerably reduced the mortality of mice in sepsis induced by the introduction of bacterial lipopolyssacharides [10]. There are also data that 8-oxo-dG is capable of activating the expression of genes of antioxidative and reparative enzymes, in particular, of DNA polymerase beta, and to improve the condition of cell cultures in hypoxia, starvation and radiation [15].

In our experiments on the effect of long toxic stress caused by tetrachloromethane introduction on the process of cirrhosis we noticed abnormal decrease in 8-oxo-dG level at early stages of the experiment [16]. So, the purpose of this study was to observe the dynamics of the change in 8-oxo-dG/dG ratio in case of acute toxic stress. We used mice males of C57Bl/CBA line at the age of two months and the model of the acute toxic cirrhosis caused by tetrachloromethane introduction.

Cirrhosis is a serious illness quite often resulting in death. Histologically, it is an irreversible diffusion process characterized by the nodular transformation of parenchyma and the formation of fibrous septums connecting the portal system to the hepatic veins system. Depending on etiology, it is currently treated by analogs of bile acids (ursodiol), anti-inflammatory drugs (glucocorticoids) and immunosuppressants. The latter have serious side effects. There is reason to believe that cirrhosis development is accompanied by pronounced oxidative stress because of infiltration and inflammatory activation of immunocompetent cells and release of free radicals from microsomes, as well as ischemia and hypoxia at late stages [17]. Therefore, the content of 8-oxo-dG will increase in DNA in proportion to the severity of hepatic tissue damage. In this case, 8-oxo-dG could be a useful biomarker, both for diagnostics and assessment of the disease severity and for the subsequent determination of therapy efficiency.

The choice of a method for 8-oxo-dG determination in cellular DNA is also important. The method for 8-oxo-dG determination developed and validated in our laboratory by means of HPLC with amperometric detection [18] is exact, selective and sensitive. It is more labor-consuming than the rather widespread method of immunoenzymometric analysis. However, the accuracy of a method is more important than analysis speed upon diagnostic use of a biomarker, especially if it is necessary to observe the dynamics of the response to treatment.

Results and Discussion

The mice in our experiment were divided into two experimental groups and one control group. Toxic stress in the mice of the first experimental group was caused by intraperitoneal introduction of tetrachloromethane (1 μl/g in the form of a 30% of solution in peach-kernel oil). In order to take into account possible changes in oxidant status due to stress upon injection and to exclude possible biological effects of peach-kernel oil, a second experimental group was created. It received intraperitoneally pure peach-kernel oil in the same quantity and at the same frequency as the first group.

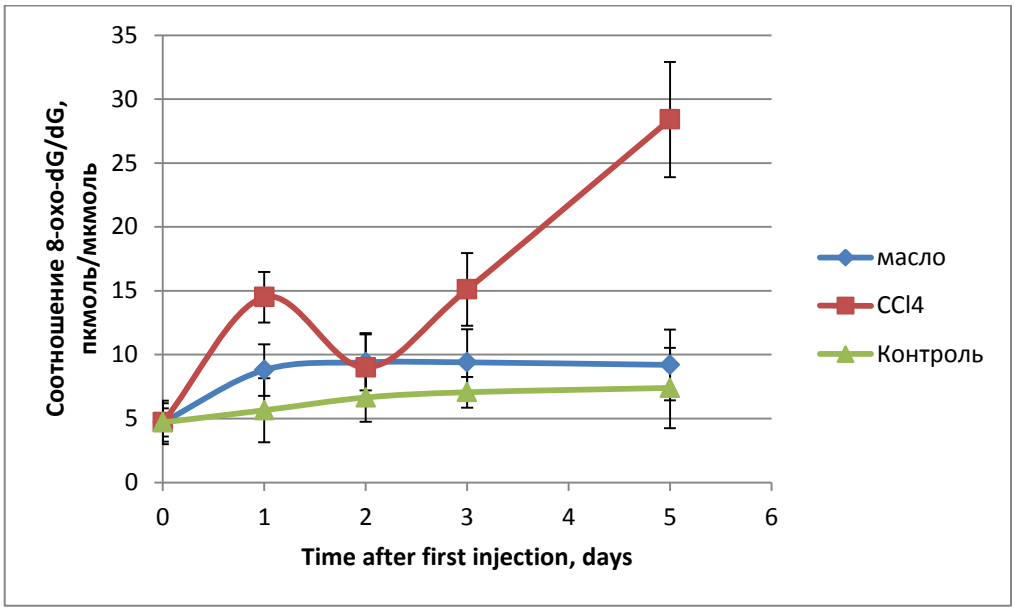
On all the days of the experiment the animals that had received oil injections showed a little higher levels of 8-oxo-dG than the intact ones. However, this difference was not always reliable. Most likely, stress due to injection is short-term, because on the third day of the experiment 8-oxo-dG/dG ratio in DNA of the mice that had received the oil injection (72 hours from the moment of intervention) approached that of the control animals. At the same time, on the first day (just 24 hours from the moment of the last injection) these mice showed doubtless increase in 8-oxo-dG content as compared to the control group.

In contrast to simple damage of tissues due to peach-kernel oil injection, introduction of tetrachloromethane exerts long-term and accumulative effect on 8-oxo-dG/dG ratio in DNA. Theoretically, pronounced oxidative stress is expected to be observed at this time. According to literature data, tetrachloromethane hepatotoxicity is caused by the fact that an active trichloromethyl radical ($CCl_3^{\bullet}$) is formed in the course of its metabolism, which damages cellular membranes and stimulates lipids peroxidation in them [19]. This results in the formation of free radicals interacting further with proteins and, finally, with nucleotides and DNA.

On the next day after the injection, the content of 8-oxo-2’-deoxyguanosine in the mice of the 1st experimental group was undoubtedly higher than in the control animals and in the animals that had received the peach-kernel oil injection (see the figure).

On the second day of the experiment (after 48 hours), the mice that had received tetrachloromethane injections showed doubtless decrease of 8-oxo-dG content in hepatic cell DNA as compared to 8-oxo-dG level obtained 24 hours after the injection (see the figure). 8-Oxo-dG/dG ratio on this day approached the value of 8-oxo-dG level of the 2nd group that had received the oil injection. These results were reproduced in three series of the experiment.

The observed decrease of 8-oxo-dG level can be explained by activation of the DNA reparation system in the course of adaptive reaction of the organism to stress. At the same time, in spite of the fact that the toxic agent is still present in the organism, 8-oxo-dG accumulation does not occur. Moreover, after 48 hours the level of oxidative damage of DNA that had increased 24 hours after the injection reverts to values almost corresponding to the control values.



Dynamics of 8-oxo-dG/dG ratio during the first 5 days of the experiment in animals that had received injections of oil and tetrachloromethane and the control group. [Соотношение 8-oxo-dG/dG, пкмол/мкмол means 8-Oxo-dG/dG ratio, pmol/μmol; масло means Oil; Контроль means Control]

It should be noted that the detected quantity of 8-oxo-dG always reflects the "equilibrium" of two processes: 2'-deoxyguanosine oxidation resulting in 8-oxo-dG formation and 8-oxo-dG reparation in DNA chain with substitution by unoxidized dG. Both processes constantly occur on a certain basic level corresponding to 8-oxo-dG content in intact healthy

animals. Respectively, we observed a pronounced equilibrium shift towards 8-oxo-dG formation during the first 24 h after tetrachloromethane introduction. Its content increased approximately 3-fold as compared to the control. It is natural and expected, if one considers the literature on mechanisms of both tetrachloromethane effect and of liver damage development under the conditions of toxic stress. However, 48 hours after the injection this shift is compensated by an increase in the intensity of a reverse process, reparation resulting in 8-oxo-dG removal from DNA. The intensity of 8-oxo-dG reparation increases in a rather short time and exceeds the level compensating its formation (in this case the curve would show a plateau or a small dip) and reaches a higher value capable of shifting the equilibrium in the opposite direction. Such a considerable and rapid intensification of reparation is hardly possible without a certain signal resulting either in an increase of 8-oxyguanine-glycosylase-1 (OGG-1) expression or in an increase of its activity at the invariant number of copies of the enzyme, or in the start of alternative reparation mechanisms usually not applied to remove 8-oxo-dG from the DNA of a nonproliferating cell. Perhaps even 8-oxo-dG itself or a similar product of biomolecules oxidative damage can serve as such a signal for the induction of adaptive response of the cell. A number of both theoretical and experimental data indicate that 8-oxo-dG could act as a regulator of adaptive activation of reparation. In particular, its precursor, 2'-deoxyguanosine is present in the cell in large quantities, not only as a part of DNA difficult to reach for free radicals, but also in the cytoplasmic pool, and it has a rather low redox potential. 8-oxo-dG itself is a small and mobile molecule, which makes it similar to many signal compounds. Importantly, 8-oxo-dG is easily exposed to further oxidation, and most likely it does not live long under the conditions of active intracellular environment [20]. This provides a rapid termination of the signal when it is no longer required. Lastly, this version is confirmed by the experimental data reporting about activation of some reparation enzymes with exogenous 8-oxo-dG or with a mixture of oxidized nucleotides, as well as about their common protective and adaptogene action [21].

After a repeated injection of tetrachloromethane, on the third day of the experiment 8-oxo-dG/dG ratio in the DNA of animals of the first experimental group increased again as compared to intact control. Most likely, the repeated introduction of tetrachloromethane results in compensation threshold crossing, so, the reparation and antioxidant protection systems are not capable any more to overpower the increasing oxidative damage of DNA and of the cell in general.

By the fifth day of the experiment the content of 8-oxo-dG in DNA of the hepatic cells of the first experimental group mice was more than 5-fold higher than that both in the intact animals and in the second experimental group. Apparently, at such duration and intensity of toxic stress its compensation by means of any reparation and antioxidant systems available in the cell became impossible. In experiments of our colleagues that used a similar scheme [22] it was on the fifth day that pronounced histologic symptoms of toxic cirrhosis were found. This clearly demonstrates that the adaptive potential of the cell becomes exhausted by this moment. After this, tissue reactions follow: inflammation, fibroidization, infiltration of macrophages and other attempts, to a greater or lesser degree successful, of overpowering the increasing tissue damage.

It can be noticed that the mechanical stress due to oil injection does not give accumulative effect: the biomarker level on the fifth day shows again the doubtlessness of the difference as compared to the initial point and the intact control group, but on the average it does not differ from that on the second and third experimental days.

Thus, it was shown that short-term toxic stress results in a rapid increase of 8-oxo-dG level in DNA. (This level decreased to the level of the control values 48 hours after the first injection.) Such behavior of the biomarker can be explained by activation of the reparation and antioxidant systems. Subsequent injection resulted again in an increase of 8-oxo-dG level. At a later stage this level only increased, which indicates exhaustion of the organism reparation potential of and leads to the development of inflammation and a pathological change of the liver tissue.

Experimental

Reactants: sodium acetate, ammonium acetate (Chemmed), Tris-HCl, sodium dodecylsulfate (SDS) (Reanal), EDTA (Chemika), 2’-deoxyguanosine (Sigma Grade). 8-Oxo-2’-deoxyguanosine was synthesized similarly [23].

Ribonuclease A 10 mg/μm (Qiagen), nuclease P1 100U lyophilized from a mixture of 20 mM sodium acetate, pH 5.3, 1 mM $ZnCl_2$, 50 mM NaCl (USBiological), proteinase K, 100 μg/ml (Fermentas) alkaline phosphatase 1 unit/μl dissolved in 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$.

Acetonitrile HPLC-R (Biosolve), ethanol (SERVA), chemically pure chloroform, isoamyl alcohol (Chimmed).

Buffer solutions

Buffer I: 150 mM NaCl, 10 mM Tris-HCl, EDTA (pH 8.0).

Buffer for nuclease PI: 20 μl of 10 mM $(AcO)_2Zn$, 40 μl 0.1 M of AcONa.

Buffer for alkaline phosphatase FastAP™: 10 X 100 mM Tris-HCl (pH 8.0 at 37°C), 50 mM $MgCl_2$, 1M KCl, 0.2% Triton X-100 and 1 mg/ml of l bull serum albumin (BSA).

Equipment: Analytical HPLC of DNA hydrolyzate samples was carried out with the use of a Beckman chromatograph (USA) equipped with two 114M Beckman pumps, a UV detector "KNAUER" with variable wavelength (detection was carried out at a wavelength of 254 nm and a cuvette thickness of 2 mm), an Altex 210A Valve “Beckman” injector, a Beckman 421A controller and an electrochemical detector made by scientific-production association “Chimavtomatika”. An ULTRASPHERE ODS column (5 microns, 4.6*250 mm, Beckman) was used. UV spectra were registered with the use of a Helios α "Unicam" spectrometer (Great Britain) in the range of wavelengths 220–300 nm. Samples were precipitated in a Sigma 202 MK "Sigma" centrifuge (USA) and dried in a Speed Vac Concentrator "Savant" device (USA).

DNA concentration was determined by spectrophotometry at λ = 260 nm; dG at λ = 254 nm, 8-oxo-dG at λ = 293 nm.

Chromatograms were registered by means of Multikhrom software ("Ampersand Ltd.", version 1,52u, Russia).

Model of acute toxic liver damage

Mice males of C_{57}Bl/CBA line at the age of 2 months were used in the experiment. The animals were kept in a vivarium at free access to water and food, with natural change of day and night, t=20 °C.

The mice were divided into three groups: two experimental groups and a control group. Toxic stress was caused in the first experimental group animals by CCl_4 intraperitoneal introduction (1 μl/g in the form of a 30% solution in peach-kernel oil) at the beginning of the first and third days of the experiment. The animals of the second experimental group received intraperitoneally pure peach-kernel oil in the same quantity and at the same frequency as those of the first one. In order to study the dynamics of oxidative stress development the animals were taken out of the experiment on different times: on the 1st, 2nd, 3rd and 5th days after its beginning. The liver of the mice was used for DNA isolation and 8-oxo-dG/dG ratio measurement.

Isolation of nucleic acids from mice liver

Mice liver samples (about 100 mg) were placed in plastic test tubes for centrifugation. After this 400 μl of a buffer (150 mM NaCl, 10 mM Tris-HCl, EDTA (pH 8.0)) was added, and the samples were homogenized. Then 20 μl of 20% SDS and 40 μl of proteinase K (c =10 mg/ml) were added to 400 μl of the obtained homogenate, and the samples were incubated for 2 h at 55° C. After the incubation 3M $LiClO_4$ (40 μl) and 3M sodium acetate (40 μl) were added, and the mixture was stirred by shaking.

Chloroform – isoamyl alcohol mixture (24:1, 600 μl) was added to the obtained solution. The obtained mixture was stirred and centrifuged at 13,400 rpm for 10 min. The aqueous layer was separated. This procedure was repeated twice. Then 96% ethanol (1.5 ml) cooled to –18°C was added to the water mixture. The samples were kept overnight at –20°C and centrifuged at 8,000 rpm for 10 min. Ethanol was decanted, and 75% ethanol cooled to –18° C was added to attain a volume of 2 ml. The samples were stirred in a "Vortex" device, and then centrifuged at 13,400 rpm for 10 min. The ethanolic solution was discarded. The obtained precipitate of nucleic acids was dried for 30 min in vacuum and stored at –18°C.

DNA isolation

The precipitate obtained at the previous stage was dissolved in 200 μl of a buffer (150 mM NaCl, 10 mM Tris-HCl and EDTA (pH 8.0)). Ribonuclease A (1 μl, 10 mg/ml) was added, and the mixture was incubated in a thermostat at 37°C for 4 h. After the incubation 96% ethanol (1.8 ml) cooled to –18°C was added into each test tube. The mixture was left overnight at –20°C and centrifuged at 8,000 rpm for 10 min. Ethanol was decanted, and 75% ethanol cooled to –18°C (1 ml) was added. The mixture was stirred and centrifuged at 13,400 rpm and –30°C for 5 min. Ethanol was decanted, and the samples were dried. The precipitate was dissolved in 400 μl of distilled water. DNA concentration was determined by UV spectroscopy, and aliquots containing 10 relative units were selected into test tubes for subsequent hydrolysis. The samples were dried in a Speed Vac Concentrator for 30 min. The dried samples were stored at –80°C.

DNA hydrolysis

The samples obtained after extraction were dissolved in 200 μl of distilled water. A buffer for nuclease PI (20 μl) was added to the obtained solutions. The solutions were heated for 3 min at 95°C and quickly cooled. Active PI nuclease (2.0 units) was added, and the mixture was

incubated at 55°C for 2 h. A buffer for alkaline phosphatase (22 μl) and alkaline phosphatase (1 μl, 1 act. un.) were added. The mixture was incubated at 37°C for 1 h, heated to 80°C for 5 min and centrifuged for 5 min at 13,400 rpm.

Reversed-phase HPLC combined with amperometric detection (for DNA hydrolyzate)

The obtained hydrolyzate of DNA was analyzed by means of RP-HPLC with the use of spectrophotometric and amperometric detection. Buffer A: 0.1M $AcONH_4$. Buffer B: CH_3CN. Gradient: 5–15% of acetonitrile. Column: ULTRASPHERE ODS, 4.6 mm × 250 mm (5 μm). Detection was carried out with the use of a UV detector at 254 nm and of an amperometric detector in the constant mode at an electrode voltage of 0.4 V.

Acknowledgment

We are grateful to the staff member of the Embryology department of the Biological faculty of Lomonosov Moscow State University Suprunenko E.A. for providing biological material for the study.

References:

1. Nevredimova T.S., Marmiy N.V., Esipov D.S., Esipova O.V., Shvets V.I. Vestnik MITHT (Fine Chemical Technologies). 2014. V. 9. № 5. P. 3–10. (in Russ.).
2. Roszkowski K., Jozwicki W., Blaszczyk P., Mucha-Malecka A., Siomek A. Med. Sci. Monit. 2011. V. 17. № 6. P. 329–333.
3. Lisitsyna T.A., Durnev A.D. Eksperimental'naia i klinicheskaia farmakologiia (Experimantal and Clinic Pharmacology). 1999. V. 62. P. 38–41. (in Russ.).
4. Haghdoost S., Maruyama Y., Pecoits-Filho R., Heimburger O, Seeberger A., Anderstam B., Suliman M.E., Czene S, Lindholm B, Stenvinkel P., Harms-Ringdahl M. Antioxidants & Redox Signaling. 2006. V. 8. № 11-12. P. 2169–2173.
5. Svecova V., Rossner P.J., Dostal M., Topinka J., Solansky I., Sram R.J. Mutation Research / Fundamental and Molecular Mechanisms of Mutagenesis. 2009. V. 662. № 1. P. 37–43.
6. Dorszewska J., Florczak J., Rozycka A., Kempisty B., Jaroszewska-Kolecka J., Chojnacka K., Trzeciak W.H., Kozubski W. Acta Neurobiol. Exp. (Wars). 2007. V. 67. № 2. P. 113–129.
7. Hamilton M.L., Van Remmen H., Drake J.A., Yang H., Guo Z.M., Kewitt K., Walter C.A., Richardson A. Proc. Nat. Acad. Sci. USA. 2001. V. 98. № 18. P. 10469–10474.
8. Haghdoost S., Czene S., Naslund I., Skog S., Harms-Ringdahl M. Free Radical Research. 2005. V. 39. Is. 2. P. 153–162.
9. Huh J.Y., Son D.J., Lee Y., Lee J., Kim B., Lee H.M., Jo H., Choi S., Ha H., Chung M.H. Free Radic. Biol. Med. 2012. V. 53. № 1. P. 109–121.
10. Kim D.H., Cho I.H., Kim H.S., Jung J.E., Kim J.E., Lee K.H., Park T., Yang Y.M., Seong S.Y., Ye S.K., Chung M.H. Experimental & Molecular Medicine. 2006. V. 38. № 4. P. 417–427.
11. Ock C.Y., Kim E.H., Choi D.J., Lee H.J., Hahm K.B., Chung M.H. World J. Gastroenterol. 2012. V. 18. № 4. P. 302–308.
12. Hong G.U., Kim N.G., Jeoung D., Ro J.Y. J. Neuroimmunol. 2013. V. 260. № 1. P. 60–73.
13. Kim J.S., Kim D.Y., Lee J.K., Ro J.Y., Chung M.H. Eur. J. Pharmacol. 2011. V. 651. № 1. P. 218–226.
14. Hong G.U., Kim N.G., Ro J.Y. Radiat. Res. 2014. V. 181. № 4. P. 425–438.

15. Unnikrishnan A., Prychitko T.M., Patel H.V., Chowdhury M.E., Pilling A.B., Ventrella-Lucente L.F., Papakonstantinou E.V., Cabelof D.C., Heydari A.R. Free Radic. Biol. Med. 2011. V. 50. № 2. P. 270-280.

16. Nevredimova T.S., Klyus K.A., Marmiy N.V., Nalobin D.S., Chernysheva M.B., Vasilegina Yu.I., Suprunenko E.A., Bespyatykh A.Yu., Esipov D.S. Study of the protective effect of melatonin on the model of induced liver cirrhosis by analyzing the content of 8-oxo-2'-deoxyguanosine / X Int. Symp. "Biological mechanisms of aging": a collection of abstracts. May 16–19, 2012, Kharkov, Ukraine. Kharkov: Kharkov State University Publ., 2012. P. 45. (in Russ.).

17. Lieber C.S. Advances in Pharmacology. 1997. V. 38. P. 601–628.

18. Esipov D.S., Sidorenko E.V., Esipova O.V., Gorbacheva T.A., Nevredimova T.S., Krushinskiy A.L., Kuzenkov V.S., Reutov V.P. Vestnik MITHT (Fine Chemical Technologies). 2010. V. 5. № 3. P. 69–74. (in Russ.).

19. Ingawale D.K., Mandlik S.K., Naik S.R. Environmental Toxicology and Pharmacology. 2014. V. 37. № 1. P. 118–133.

20. Menshikova E.B, Lankin V.Z., Zenkov N.K., Bondar I.A., Krugovih N.F., Trufakin V.A. Oxidative stress. Pro-oxidants and antioxidants. Moscow: Slovo Publ., 2006. 556 p. (in Russ.).

21. Glebova K., Veiko N., Kostyuk S., Izhevskaya V., Baranova A. Cancer Lett. 2015. V. 356. № 1. P. 22–33.

22. Chernysheva M.B., Vasilegina Y.I., Nalobin D.S., Klyus K.A., Nevredimova T.S., Marmiy N.V., Suprunenko E.A., Bespyatykh A.Yu., Esipov D.S. Study of the protective effect of melatonin on the model of induced liver fibrosis: histologic evaluation / X Int. Symp. "Biological mechanisms of aging": a collection of abstracts. May 16–19, 2012, Kharkov, Ukraine. Kharkov: Kharkov State University Publ., 2012. P. 64. (in Russ.).

23. Satyam Nampalli, Shiva Kumar. Bioorganic & Med. Chem. Lett. V. 10. Is. 15. P. 1677–1679.