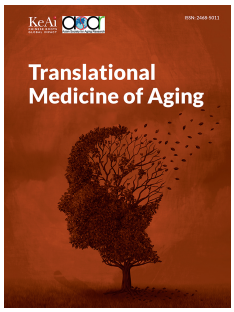


The role of metallothioneins in the formation of hierarchical mechanisms of resistance to toxic compounds in young and old animals on the example of copper sulfate

A.I. Bozhkov, V.I. Sidorov, Omar K. Alboqai, R.A. Akzhyhitov, N.I. Kurguzova, A.B. Malyshev, Mohammad Ali Yousef Albegai, Gromovoi T. Yu

PII: S2468-5011(21)00006-7

DOI: https://doi.org/10.1016/j.tma.2021.11.001

Reference: TMA 65

To appear in: *Translational Medicine of Aging*

Received Date: 13 March 2021

Revised Date: 31 July 2021

Accepted Date: 9 November 2021

Please cite this article as: A.I. Bozhkov, V.I. Sidorov, O.K. Alboqai, R.A. Akzhyhitov, N.I. Kurguzova, A.B. Malyshev, M.A. Yousef Albegai, G.T. Yu, The role of metallothioneins in the formation of hierarchical mechanisms of resistance to toxic compounds in young and old animals on the example of copper sulfate, *Translational Medicine of Aging* (2021), doi: https://doi.org/10.1016/j.tma.2021.11.001.

The role of metallothioneins in the formation of hierarchical mechanisms of resistance to toxic compounds in young and old animals on the example of copper sulfate

Bozhkov A.I.[1], Sidorov V.I.[1], Omar K Alboqai[2], Akzhyhitov R.A.[1], Kurguzova N.I.[1], Malyshev A.B.[1], Mohammad Ali Yousef Albegai[2], Gromovoi T. Yu[3]

1. Biology Research Institute V.N. Karazin Kharkov National University, 4 Pl. Svobody, 61022 Kharkov, Ukraine
2. Jerash University, Jerash, Jordan
3. Institute of Surface Chemistry, National Academy of Sciences of Ukraine, 17 Generala Naumova Vul., 03164, Kyiv, Ukraine

Abstract

We used classical methods of biochemistry, chromolithography, atomic adsorption spectrometry for the determination of copper ions and MALDI-TOF mass spectrometry for the determination of metallothioneins. We have found sequential administration of small doses of copper sulfate provided the formation of resistance to subsequent lethal doses of copper sulfate. Age-dependent differences in the adaptive response match with the difference in the metallothioneins content in liver cells. In this case of adaptation formation, there was not only an increase in metallothioneins, but also a change in the intracellular distribution of copper ions. We revealed that a multiple increase in copper ions in mitochondria and endoplasmic reticulum of liver cells was accompanied by a slight decrease in the activity of antioxidant enzymes included in their composition. It has been suggested that metallothioneins are involved not only in the accumulation and retention of copper ions, but also in active inter-protein swap. Age-dependent differences in resistance, adaptability and long-term effects of copper ions are due to differences in the metabolic systems of copper metabolism before the toxic doses administration of copper sulfate, i.e. difference in initial states.

Key words: copper resistance, age, metallothioneins, redox system.

1. Introduction

The ability to form resistance to the negative (toxic) impacts is a fundamental property of biological systems. The ability to develop resistance is influenced not only by the doses and properties of toxic compounds, but also by the age of the animals. However, the specificities and mechanisms of adaptation in animals of different ages are important in several aspects: in gerontology, since it is impossible to establish the mechanisms of aging without this knowledge; in toxicology, since induced resistance makes it difficult to interpret the results; in medical practice, since it is necessary to take into account the formation of resistance to repeated sequential doses of medication, most of which are toxic [1-4]; and even in nutritional sciences, since many essential elements in large quantities cause toxicity (in particular vitamins and copper ions) [5-6].

The problem is that studies of the mechanisms of the formation of resistance to high doses of toxic compounds can be carried out only on experimental animals with the subsequent transfer of obtained data into clinical practice. Copper sulfate was chosen as a model of the toxic factor of the environment. There were several reasons for this:

First, copper ions are an essential element and provide the activity of at least 30 enzymes in the body in physiological concentrations [7; 8]. Various pathologies appear with the complete exclusion of copper ions from the diet [9-10]. It was shown that copper ions

concentration in the body depends on age. Its content in embryos and newborns is 5-10 times higher than in adults [11]. However, the mechanisms of age-dependent changes in copper metabolism in the body have not been established.

Secondly, the intake of large doses of copper ions into the body is accompanied by the manifestation of toxicity and death of the organism [12].

Thirdly, the administration of copper salts into the organism is accompanied by its accumulation in the greatest amount in the liver, partly in the brain [13]. It makes it possible to calculate correctly the dose for young and old animals, taking into account the age-dependent changes in the mass of the target organ [14], that is, to carry out the correct gerontological experiment.

Fourthly, a violation of the copper metabolism system in the body is accompanied by the development of hepatocellular pathology – Wilson's disease with high mortality; the great practical importance is the study of the copper ions exchange features during repeated sequential administrations of copper sulfate into the body, capable of simulating this pathology [15].

Fifth, copper ions are one of the most widespread negative environmental factors.

Consequently, the study of the mechanisms of the formation of resistance in animals of different ages to copper ions is a good model in solving theoretical and practical problems of medicine. Other xenobiotics, including antibiotics, cause similar effects of the formation of biological resistance [16].

As known, a large number of specific copper-binding proteins, including metallothioneins (MTs), are involved in the exchange of copper ions in the body. It has been shown that MTs are inducible proteins and their content in the case of an increase in heavy metal ions, primarily cadmium and zinc, can increase by tens and hundreds of times [17]. The MT molecule is capable of binding from 1 to 6-8 metal atoms [18]. A hypothesis was put forward about the central role of MTs in the mechanisms of the organism adaptation to heavy metal ions [19].

Along with this, it was shown that the synthesis of MTs is induced not only by metal ions, but also by a wide range of other compounds [20; 21]. MTs belong to a large group of stress proteins [18]. In this regard, the role of MTs in the mechanisms of the formation of resistance to copper ions in animals of different ages has not been finally established. Previously, it was shown that repeated sequential administration of copper sulfate to animals leads to a number of effects, and these effects are age-dependent. They are rebalancing of the equilibrium in the prooxidant-antioxidant system towards prooxidants [22], restructuring in the immune system [23], the development of inflammatory reactions [24], and these manifestations are age-dependent [25]. Thus, there are systemic changes in the entire metabolism.

It can be assumed that in the process of the formation of the body’s resistance to toxic compounds, a hierarchical change takes place, which is based on the age-related characteristics of the distribution and binding of copper ions to various cell ligands. MTs can play a dominant role in this process.

Earlier, in a model of infection of animals of different ages with *Pseudomonas aeruginosa and Escherichia coli*, it was shown that animals of different ages use different adaptation strategies [26].

In this work, we tested the hypothesis according to which copper ions bind both to specific copper-binding proteins and nonspecific proteins (various enzymes), at the background of which MT synthesis is induced. The MTs take part in the highly dynamic transfer of copper ions to various ligands and rearrangement in regulatory systems (redox system, enzymatic

system of regulation, gene expression). As a consequence, metabolic patterns are forming capable of ensuring functioning at the background of high concentrations of copper in the cell.

To test this working hypothesis, the following was determined: the possibility of the formation of resistance in animals of different ages after repeated administrations of copper sulfate (testing of survival); intracellular distribution of copper ions in liver cells (mitochondria, endoplasmic reticulum, total cytosolic proteins and MTs); MT inducing in animals of different ages; and characterization of the redox system as one of the metabolic regulation systems in the liver of rats of different ages (the content of lipid hydroperoxides, the activity of glutathione peroxidase, glutathione reductase and aconitase).

2. Material and methods

2.1. Experimental facilities

The experiments were carried out on young (3-month-old) and old (20-month-old) male *Wistar* rats. The lethal dose of copper sulfate was determined experimentally in our laboratory and it was shown that it is 2.5-3.0 mg / 100 g of body weight or 0.58-0.69 mg / 1 g of liver for young (3-month-old) and for old (20-month-old) animals [27]. To induce an adaptive response, young and old animals were injected intraperitoneally with copper sulfate in an amount of 0.23 mg / 1 g of liver three times, with an interval between injections of 48 hours. After 24 hours after the last administration, the animals were injected with a lethal dose of copper sulfate (0.58 mg / 1 g of liver) and analyzed the survival rate according to Kaplan-Meier [28].

To determine biochemical parameters (Fig. 1), the animals were anesthetized with ether and decapitated 24 hours after the last threefold administration of copper sulfate in an adaptive dose (0.23 mg / 1 g of liver) (Fig. 1). Blood samples were collected after decapitation and used to obtain serum.

The samples were kept in dry test tubes at a temperature of 15–20°C for 30 min, centrifuged at 1000 g for 15 min, and serum was obtained.

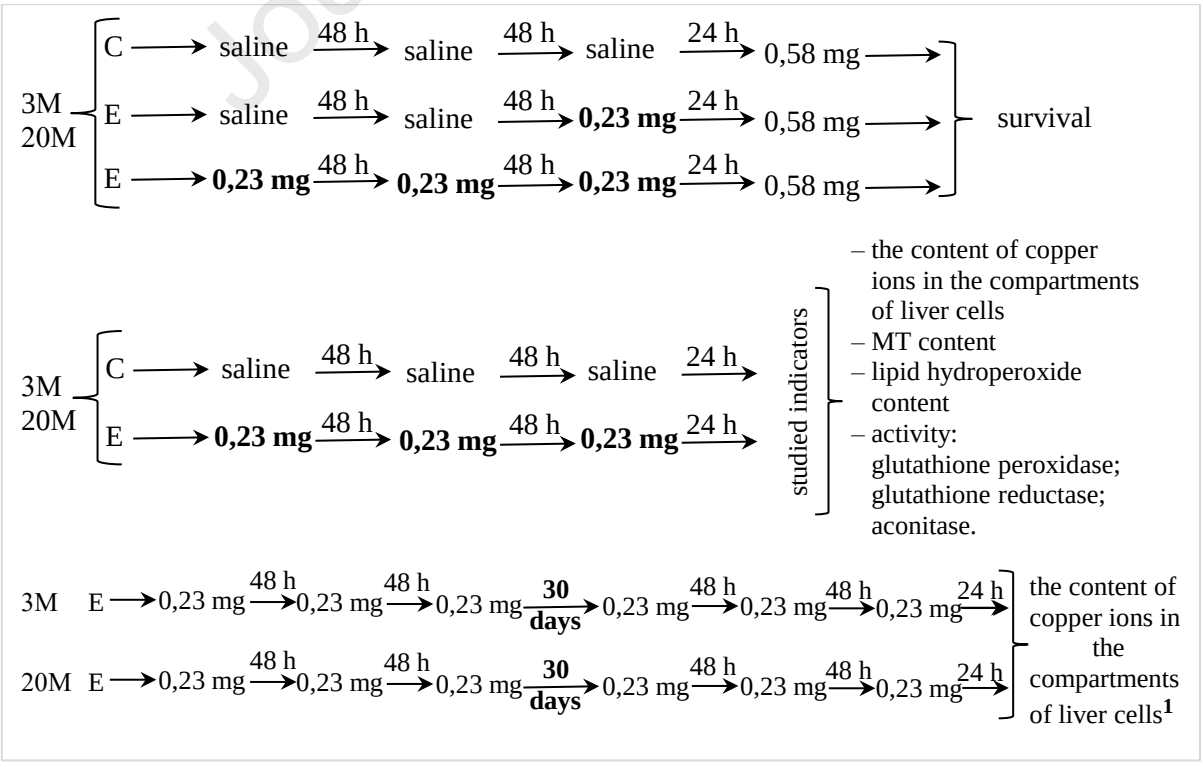


Fig. 1. Scheme of the sequence administration of a copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in different doses (indicated by arrows, the adaptive dose is 0.23 mg / 1 g of the liver and the lethal dose is 0.58 mg / 100 g of body weight) to young (3 months old – 3M) and old (20-month-old – 20M) experimental animals (E) with an interval between administration of 48 hours. The control group (C) of animals received saline.

1 – the content of copper ions in the compartments of liver cells after its excretion after 30 days and repeated three-fold administration at a dose of 0.23 mg / 1 g of liver.

2.2. Fractionation of liver cells

The liver (equal samples of 8 grams) were pressed and mixed with 100 mM tris-HCl buffer solution (pH 7.4) containing 250 mmol of sucrose, 5 mmol of KCl, and 1 mmol of MgSO4. The mixture was homogenized at 800 rpm for 1 min and filtered through a piece of nylon fabric.

Cytosol, mitochondria (electron microscopy was used to control the purity of the fractions) and endoplasmic reticulum (ER) (marker enzyme glucose-6-phosphatase was used as a control of purity) were isolated by differential centrifugation as described at [29].

2.3. Determination of low molecular weight proteins from cytosol

The liver cytosol was isolated by differential centrifugation after precipitation of mitochondria, membranes, and ER was purified by gel chromatography on a Sephadex G-75 column (diameter 25 mm, gel height 400 mm). 30 g of Sephadex was suspended in Tris-HCl buffer (pH 7.4) and put on onto the column. The protein eluate was collected at a rate of 30 ml / hour. Fractions of 3 ml were collected into test tubes.

The protein content in the fractions was determined spectrophotometrically at 260 and 280 nm (on a SHIMADZU UV-2600 spectrophotometer, Japan), and copper – in the same way as the content of copper ions in the cytosol fractions, low molecular weight proteins of the cytosol (LPC), ER, and mitochondria – an atomic adsorption spectrophotometer (s-115m1, AO "SELMI", Ukraine). We calculated the amount of copper per mg of protein for the corresponding fractions.

The eluates of the liver cytosol of experimental animals, which contained the highest amount of copper in comparison with other eluates of this group of animals, were attributed to the LPS fraction containing MT, and were also translated into the eluates of other groups of animals. Subsequently, the concentrate of these proteins was separated on a mass spectrometer by the MALDI-TOF method (point 3.1).

$CuSO_4 \cdot 5H_2O$ was additionally added to the obtained concentrates of these proteins in the *in vitro* system with a copper concentration of 0.25 mg / ml for MALDI-TOF separation to assess the specificity of the binding of copper ions to proteins (LPC).

3. Analytical methods

3.1. LPCs mass spectrometry

The LPCs isolated by gel chromatography were further investigated by the MALDI-TOF method. The samples were concentrated using a rotary evaporator and brought to the same protein concentration in the sample of 1.5 mg/ml. Mass spectrometric studies were performed on an Autoflex II LRF 20 Bruker Daltonics equipped with a pulsed nitrogen laser (λ = 337 nm, pulse length 3 ns). The mixed with the matrix samples were applied to a standard steel target and dried at room temperature. Each mass spectrum is represented by the sum of 490 individual spectra. The studies were carried out in a linear device mode of operation with the positive and negative ions detection. The matrix for mass spectrometric analysis was prepared according to

standard procedures: 12 mg of sinapic acid (Fluka) was dissolved in 1 ml of a 1:1 (v/v) mixture of water-isopropanol alcohol with the addition of 0.1% trifluoroacetic acid. The obtained results analysis was carried out using the open software ProteoWizard (http://proteowizard.sourceforge.net), mMass (http://www.mmass.org) и OpenMS (https://www.openms.de).

3.2. Determination of the lipid hydroperoxides (HPL)

The mitochondria swelling were recorded by changing the optical density in a thermostated (37°C) cuvette with constant agitation by spectrophotometer Specord UV VIS (Germany) at 610 nm. The incubation medium composition was the following: 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM KH_2PO_4, 5 mM rotenone, 2 mM succinate and 25 mM $CaSO_4$. The lipid hydroperoxide (HPL) content in liver microsomes and mitochondria was determined by means of the Ohkawa et al. method [30]. The HPL content in serum was determined as previously described in [31]. The absorption spectrum of the colored product was recorded on double-beam spectrophotometer Specord UV VIS, measuring the difference in extinction at 535 and 520 nm [32]. The HPL content was expressed in equivalent amounts of using a molar extinction coefficient of $1.56 \times 105 \times M^{-1}$ cm^{-1}. The activity was expressed in nmol MDA/mg protein.

3.3. Glutathione peroxidase activity

Glutathione peroxidase activity (GPx, CP 1.11.1.9) was determined in cytosolic fractions, and liver mitochondria by spectrophotometry at 340 nm with the help of the method of [33] in 50 mM K^+, Na^+ phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.15 mM NADPH, 1 unit of yeast glutathione reductase, 0.2% Triton X-100 and 3 mM Na azide to inhibit KAT. 1.2 mM cumene hydroperoxide and 0.4 mM hydrogen peroxide were added. Incubation temperature was 37°C. The activity was expressed in nmol NADPH/min per mg of protein or ml of serum considering a molar extinction coefficient $6.22 \times 103 \times M^{-1}$ cm^{-1}.

3.4. Activity of glutathione reductase

The activity of glutathione reductase (GR EC 1.6.4.2) in liver mitochondria was measured spectrophotometric by decrease of NADPH [34] in a medium containing 50 mM K^+ phosphate buffer, pH 7.4, 1 mM EDTA, 0.16 mM NADPH, 1 mM GSSG, 0.2% Triton X-100. The incubation temperature was 37°C. The activity was expressed in nmol NADPH/min · mg protein with consideration of the coefficient of molar extinction $6.22 \times 103 \times M^{-1}$ cm^{-1}.

3.5. Activity of aconitate hydratase

The activity rate of aconitate hydratase (aconitase, EC 4.2.1.3) was measured according to the procedure described in [35] and expressed in nmol of aconitate per 1 mg of proteins using the molar extinction coefficient 3.6 mM^{-1} cm^{-1}.

3.6. Total protein content

The content of total protein in samples was assayed using the method of Lowry et al. [36].

3.7. Bioethical standards

Experiments for laboratory animals to assess the effect of copper sulfate were carried out in agreement with the bioethical committee of V.N. Karazin Kharkov National University, which is guided by the provisions of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, March 18, 1986) and "International Recommendations (Code of Ethics) for the conduct of biomedical research using animals" (Council of International scientific organizations, 1985).

3.8. Statistical analyses

The mean, standard deviation, standard error of the mean, and sample size were used as characteristics of the obtained samples. The statistical significance of differences between data groups was assessed using the nonparametric Mann-Whitney U-test. Statistical processing of the results was carried out using the "Excel" program. Differences between the data of the control and experimental variants were considered significant at $p < 0.05$.

4. Results

4.1. Induction of resistance to lethal doses of copper sulfate in young and old animals

Intraperitoneal injection of copper sulfate to young rats (3 months) at a dose of 2.5 mg / 100 g of body weight (or 0.58 mg / 1 g of liver weight) led to the death of 50% of the animals in 4 hours after administration, and by 72 hours all animals of this group died (Fig. 2.A, young). If copper sulfate was administered to old animals (20 months) at the same dose – 0.58 mg / 1 g of liver weight, then the death of the animals occurred much later: by the 48th hour of the experiment, 80% remained alive, by the 72nd hour the survival rate was 20% old animals (Fig. 2.A, old). However, the statistical power was not enough to reject the null hypothesis, so at this stage it cannot be argued that old animals showed greater resistance to a lethal dose.

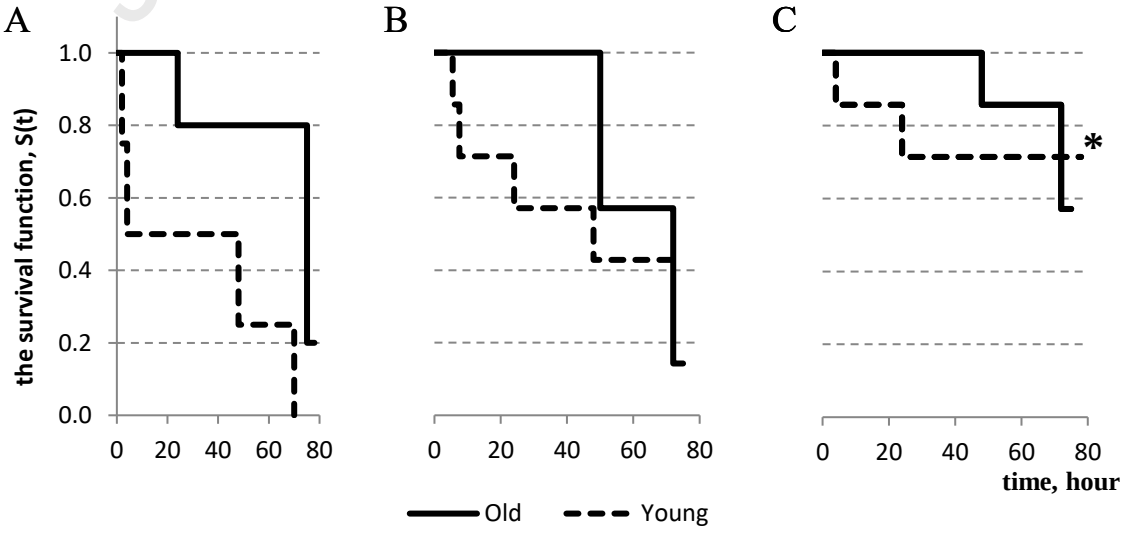


Fig. 2. A – the number of animals that survived according to Kaplan-Meier (assessment of the survival function) after the administration of copper sulfate in a lethal dose (0.58 mg / 1 g of liver weight) in the case of young animals and old animals (7 animals per group); B – the number of animals that survived after a preliminary single administration of a small adaptive

dose (0.23 mg / g liver) of copper sulfate, and after 24 hours the same animals received a lethal dose for young and old animals, respectively (7 animals per group); C – the number of animals that survived after 3 preliminary injections of a small adaptive dose and, accordingly, a lethal dose for young and old animals (7 animals per group). * – significant differences $p < 0.05$ in young animals receiving three adaptive injections (Fig. C) in comparison with their control (Fig. A) (log-rank criterion).

In order to study the ability of young and old animals to adapt to the action of copper sulfate, the animals were preliminarily administrated with copper sulfate in small doses (0.23 mg / 1 g of liver weight), followed by the administration of a lethal dose of copper sulfate (0.58 mg / 1 g liver). So, it turned out, that the temporal nature of the induced resistance depended on the age of the animals: in the old, death occurred later than in the young, but at the same time, after 48 hours, it was on the contrary more (Fig. 2.B). However, it is again impossible to talk about significant differences at this stage.

Finally, after three times, the administration of small doses of copper ions (Fig. 2.C) followed by the administration of a lethal dose in the group of young and old animals by the third day, a well-pronounced effect of resistance to a lethal dose of copper sulfate (hormesis) was manifested. Thus, 71% survived in the young group and 57% in the old group, but we can speak about the reliability of the data obtained only for young animals (Fig. 2.C).

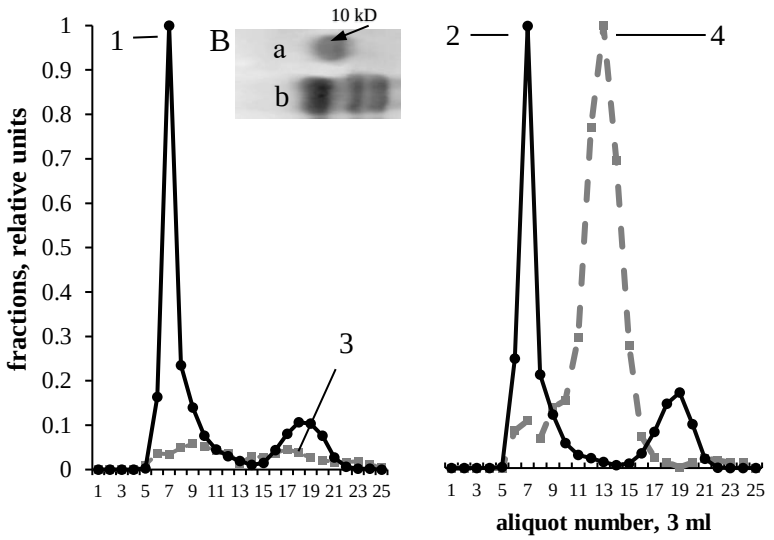
It is also important to note that the introduction of adaptive doses (single and triple), which amounted to 40% of the lethal dose, did not lead to the death of animals, both during the setting of the survival scheme for this part of the work, and during the setting of other schemes with the introduction of copper sulfate described in this work (Fig. 1).

These results allow us to conclude that repeated preliminary administration of small doses of copper sulfate induces the formation of resistance to subsequent lethal doses. At the same time, death in a group of young and old animals may have a different temporal character, which suggests that death in different age groups was caused by different reasons and, possibly, old animals used other strategies of adaptation to the action of this toxicant; and this statement is consistent with the data on the distribution of copper ions (section 4.2) and mass spectrometry of proteins of the liver cytosol of experimental animals (section 4.3) described in this work.

4.2. Intracellular distribution of copper ions in animals of different ages with various schemes of copper sulfate administration.

Low molecular weight proteins (LPS) were isolated from liver cytosol fractions. So, if the animals were previously injected three times with copper sulfate in an adaptive dose (for the example of young ones), then when comparing the concentration of copper in the obtained fractions, it was possible to observe its increase with a maximum in aliquots No. 11-15 (Fig. 3.A, curve 4). At the same time, such an increase was not observed in intact animals (Fig. 3.A, curve 3). The proteins that were in these aliquots and whose distribution was identical in both the experimental and the intact group (Fig. 3.A, curves 1 and 2) had a molecular weight (MW) of no more than ~10 kDa. This fraction of proteins was summarized as LPS, and judging by the concentration of copper in the experimental animals, it was assumed that it contained metallothioneins (MTs).Subsequently, aliquots with a fraction of these proteins were analyzed by the MALDI-TOF method (section 4.3).

A – chromatographic profile



1 – protein fractions (young, intact)
2 – protein fractions (young, exp-tal)
3 – copper ion content (young, intact)
4 – copper ion content (young, exp-tal)

C – copper content (intact)

Fractions liver	Young		Old	
	mcg / mg protein	%	mcg / mg protein	%
LPC	**0,62**±0,01	81,5	**0,64**±0,091	83
Cytosol	**0,05**±0,009	6,5	**0,06**±0,009	7,9
ER	**0,05**±0,008	6,5	**0,03**±0,009	3,8
Mitochondria	**0,04**±0,006	5,2	**0,04**±0,005	5,1

D – copper content (three injections)

Fractions liver	Administration scheme			
	Young		Old	
	Three times injections of copper sulfate mcg / mg protein	%	Three times injections of copper sulfate mcg / mg protein	%
LPC	***5,30**±0,09	61	****6,00**±0,09	65,6
Cytosol	***0,42**±0,08	4,8	****0,40**±0,08	4,4
ER	***2,10**±0,1	24	****1,80**±0,1	19,7
Mitochondria	***0,84**±0,08	9,7	****0,94**±0,2	10,3

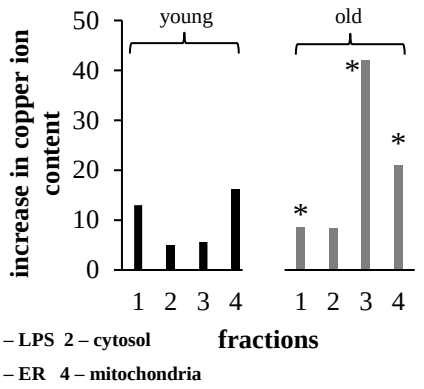
Fig. 3. A – chromatographic profile (Sephadex G-75) of the liver cytosol, normalized to the maximum copper concentration, which were obtained in intact young animals (1) and animals after three consecutive administration of copper sulfate at a dose of 0.23 mg / 1 g of liver weight (2); as well as the content of copper ions per mg of protein in these fractions in intact animals (3) and animals after three consecutive administration of copper sulfate at the same dose (4); B – electrophoretic separation of these proteins that bound copper ions (fractions 11-15) were separated in PAGE: marker protein (a); experimental sample (b). C is the content of copper ions in the intact liver fractions of young and old animals; D – the content of copper ions in the liver fractions of young and old animals 24 hours after three injections of small doses of copper sulfate (0.23 mg / g liver) with an interval of 48 hours after the administration. Each group contained 3 animals for the isolation of proteins for chromatography and 5 animals in a group for biochemical studies. */** – the variants are marked in which $p<0.05$ in young/old experimental animals (Fig. D) in comparison with their intact level (Fig. C).

In order to determine the specificity of the binding of copper ions to the isolated LPC, the content of copper ions in the cytosol, ER and mitochondria of the liver was determined. It was found that in intact young and old animals, 81.5-83.0% of copper ions are localized in the LPC, and from 4 to 8% of copper ions are associated with proteins of the cytosol, ER and mitochondria (Fig. 3.C). In the event that young animals were administrated three times with copper sulfate, then only 61% of copper ions were associated with LPC, 24% in ER, almost 10% in mitochondria, and 4.8% with cytosolic proteins, and this did not depend on age (Fig. 3.D). Consequently, after a three-fold sequential administration of copper sulfate to animals, which was accompanied by the induction of resistance to lethal doses of copper, the preferential binding of copper to LPC was "disrupted" and it bounded in large quantities with ER, mitochondria and other proteins, that is, no specific or preferential increase in the content of copper ions in these fractions was observed.

When comparing the degree of increase in copper ions after three consecutive injections of adaptive doses of copper sulfate in comparison with intact animals, it turned out that the

pattern of copper distribution in the studied fractions was different in young and old animals. (Fig. 4.A).

A – increase of Cu^{+}



B – copper content after 30 days

Fractions	Young	Old
	mcg / mg protein	mcg / mg protein
LPC	**0,63**±0,12	**0,63**±0,1
Cytosol	**0,039**±0,01	**0,034**±0,01
ER	**0,05**±0,01	**0,040**±0,01
Mitochondria	**0,044**±0,008	**0,037**±0,009

C – copper content after 30 days (re-introduction)

Fractions	Young	Old
	mcg / mg protein	mcg / mg protein
LPC	**15,3**±0,91	***30,0**±0,85
Cytosol	**0,205**±0,08	***0,877**±0,05
ER	**0,250**±0,08	***0,61**±0,09
Mitochondria	**0,475**±0,07	***1,764**±0,10

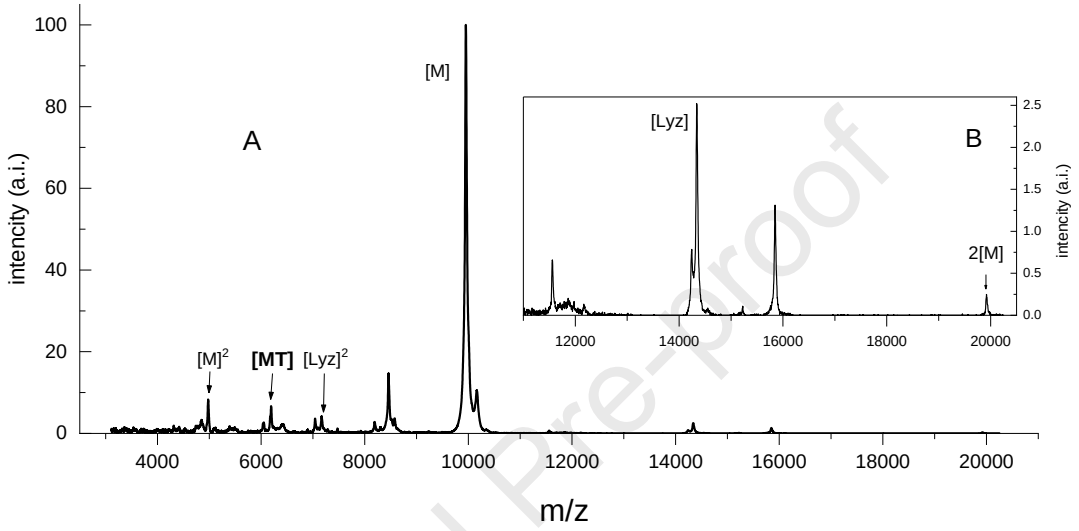
Fig. 4. A – an increase in the content of copper ions in different fractions of liver cells: LPC (1), cytosol (2), ER (3) and mitochondria (4) after three consecutive administration of copper sulfate in an adaptive dose of 0.23 mg / 1 g liver compared with the initial (intact) level in young and old animals, respectively, B – the content of copper ions in the studied fractions in young and old animals a month after three consecutive administrations of copper sulfate, C – the content of copper ions in these fractions of liver cells, young and old animals, which were administrated three times with small doses of copper sulfate, and 30 days later they were re-administered the same adaptive doses of copper sulfate (re-introduction). * – variants $p<0.05$ were noted in old experimental animals compared with young experimental animals against the background of repeated administration of copper sulfate after 30 days (re-induction).

The characteristic of the response to repeated influences of the same factor in some time after the primary influence (long-term consequences) is of great interest in the study of adaptation mechanisms. It was found that 30 days after three consecutive injections of copper sulfate, its content in the liver fractions did not differ from the initial level (Fig. **3.C** – intact level, **4.B** – 30 days after three injections of $CuSO_4*5H_2O$). That is during this time, copper ions were completely removed from the body. If the same animals were again administrated three times with copper sulfate (that is, the second cycle of influences) (Fig.**4.B**), then its content in the LPC was increased by 2.9 times in young animals compared to the first cycle of administration (Fig. **3.D** ; **4.B**, young), and in old animals, the content of copper ions in LPC was 2 times higher than in young animals and 5.6 times higher than their intact level (Fig **3.C**; **4.B,** old), and amounted to 30 μg Cu^{2+} per mg protein. At the same time, the content of copper ions in the fraction of cytosol, ER and mitochondria in young animals, on the contrary, was reduced by 2.2; 4.4; and 1.8 times in comparison with the first cycle of copper sulfate administration, while in old ones, on the contrary, it increased 2 times in the cytosol and mitochondria, and decreased 3 times in ER, i.e. the intracellular distribution pattern of copper ions is changed (Fig. 3.C,D; 4.B.C). Consequently, after repeated administration of copper sulfate in young animals, 94% of copper ions were bound to LPC, and 90% in old animals, i.e. there was an increase in the degree of "specificity" of the binding of copper ions with LPC in comparison with the first cycle of injections (81.5% and 83.0%). Despite such a high content of copper ions in the fractions, it had no significant changes in the functional properties of the liver.

355

356 *4.3. Characterization of liver metallothioneins in intact and adapted to copper ions in young and*
357 *old animals.*
358

359 The LPC isolated by gel chromatography were further investigated by the MALDI-TOF
360 method. The obtained spectra have common features: the most intense peaks are in the range
361 from 5000 to 10000 m / z. The intensity of the peaks in the 11,000-20,000 m / z range is much
362 lower, so they are shown separately at 40x magnification (Fig. 5.A; B).
363



364 **Fig. 5.** A – general view of the spectra with the identification of the main peaks of the
365 LPC. The main ion [M], it's doubly charged ion [M] 2, singly charged dimer 2 [M], lysozyme
366 [Lyz] (m = 14332 Da) for additional calibration, its doubly charged ion [Lyz] 2. [MT] – region
367 with 6200 m / z – metallothioneins, B – part of the spectrum in the region 11000 – 20000 m / z
368 increased 40 times. A typical curve from three independent experiments is shown.
369

370 So, at this stage of the study, we were interested in studying the interaction of proteins
371 (LPS) with metals against the background of the introduction of adaptive doses of copper sulfate
372 (40% of the lethal dose). In the case of the formation of a protein compound with one or more
373 copper atoms, the resulting complex will appear on the MALDI spectra in the form of a
374 sequential peak/s located to the right of the main peak, which will differ from it by the mass of
375 one or several copper atoms (its mass is about 60 Da). Such a structure with a series of regularly
376 repeating peaks was found in the range of 6000-6600 Da for the spectra of young experimental
377 animals receiving adaptive introduction of copper (complexes from 1 to 5 copper atoms are
378 shown) (Fig. 6.C).
379
380

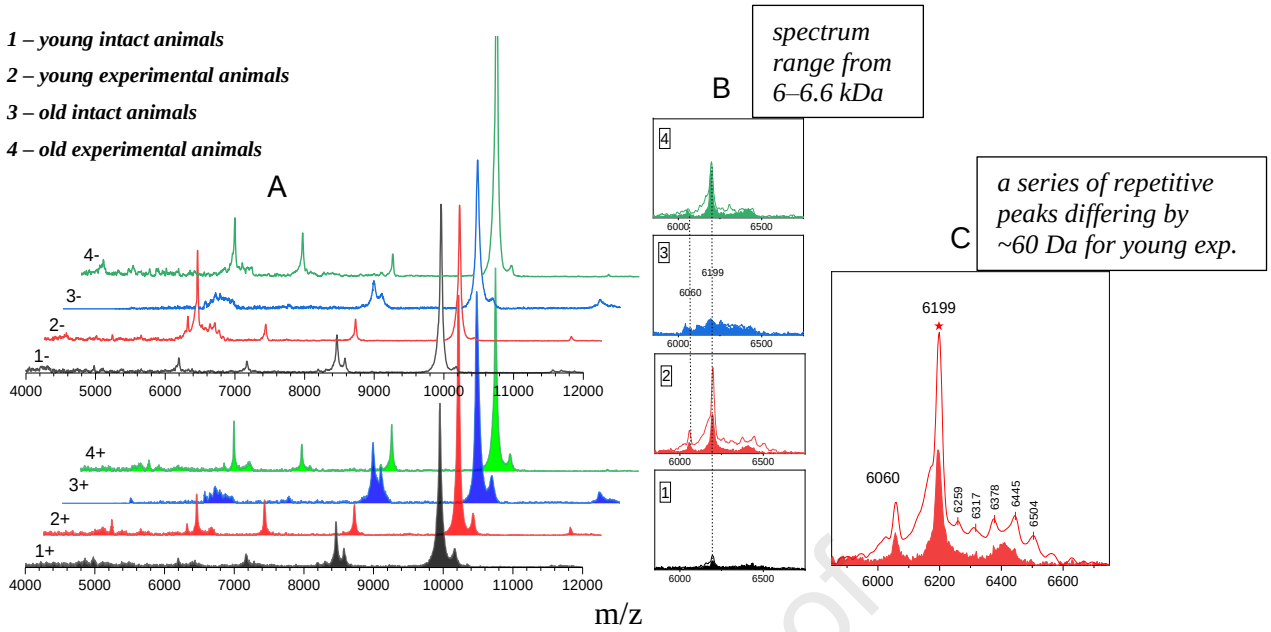


Fig. 6. Individual MALDI-TOF spectra of low molecular weight proteins of the liver cells` cytosol in the registration mode of positive (+, curves with filling) and negative (-, curves without filling) ions: A – young intact animals (1), old intact animals (3), young animals that were administrated three times with copper sulfate at a dose of 0.23 mg / 1 g of liver (2) and old animals, which were administrated with copper three times at a dose of 0.23 mg / 1 g of liver (4), respectively, in the case of positive (+) and negative (-) registration of spectra. B – the region of individual MALDI-TOF spectra with the main peak m / z = 6200, assigned to metallothioneins, respectively for options 1-4. C – spectrum of metallothioneins and its complexes with copper atoms for young rats treated with copper sulfate three times at a dose of 0.23 mg / g liver. A typical curve from three independent experiments is shown.

The main peak falls on a protein with a MW of 6200 Da, which falls within the range of masses expected for specific metal-binding proteins [17; 18]. Thus, based on the above, we can classify the 6200 Da protein as MT, while a comparative analysis of the spectra in different experimental groups shows that in intact young animals the peak at 6200 m / z corresponding MT is weakly expressed (Fig. 6.B.1). At the same time, in intact old animals, the content of this protein was presented in a significantly greater amount than in young ones (Fig. 6.B.3). Consequently, MTs are present in intact animals and their content in old animals exceeds those in young ones before the administration of copper sulfate, i.e. the initial MTs level is higher in old animals than in young ones. In young animals receiving triple copper sulfate, which was accompanied by the induction of resistance to copper ions, the protein content with a molecular weight of about 6200 Da increased many times compared to the intact level, that is, induction of the synthesis of these proteins took place (Fig. 6.B.2). Their content also increased in old animals after the administration of copper sulfate (Fig. 6.B.4), although to a lesser extent. Consequently, the formation of resistance to copper sulfate was accompanied by the induction of MTs synthesis in young and old animals. However, it is not clear what their contribution to the mechanisms of induced stability.

As seen in Fig. 6.C MTs with a MW of about 6200 Da are capable of binding five or more copper atoms. It is known that MTs can bind metal ions based on two mechanisms: cooperative and non-cooperative. In the first case, it is assumed that the affinity for metals in MTs increases with each attached atom, and in the second case, each atom is attached independently of the others, i.e., previously bound to MTs [18]. In the case of the cooperative mechanism of MTs binding to copper, the equilibrium will be shifted towards filling a larger number of MTs binding sites with metal – up to seven or eight with a sufficient amount of

copper in the medium, and such binding should be practically irreversible. The affinity for copper atoms would be very high, several orders of magnitude higher than the affinity for copper for other proteins. Such complexes on the MALDI-TOF spectra can be recorded in the range 6500-6700 m / z (Fig. 7.B.MT+8Cu), however, this was not observed in real spectra (Fig. 6.B).

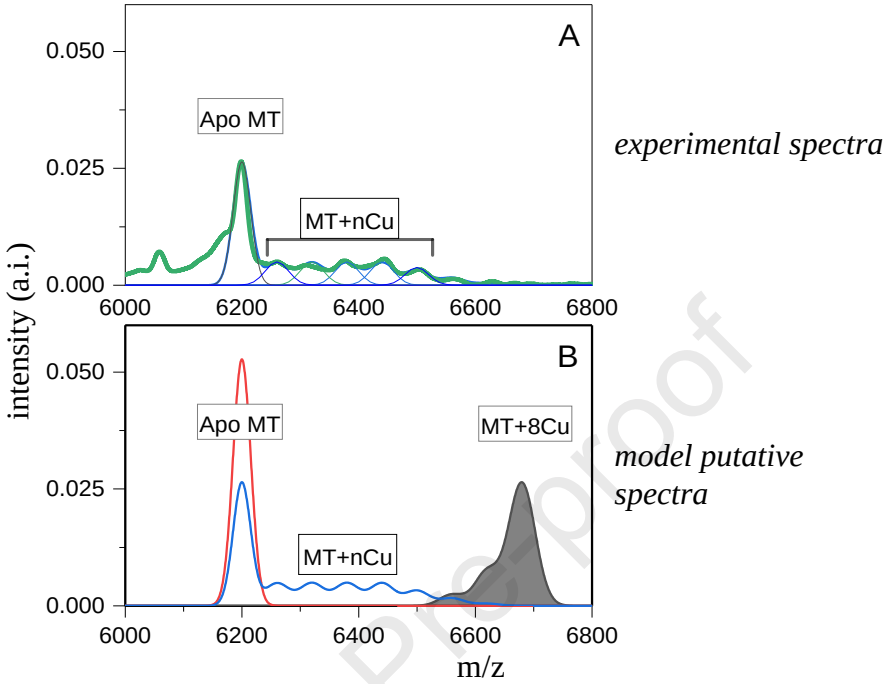


Fig. 7. Examples of MALDI-TOF spectra for different models of MTs binding to copper. A – experimentally obtained MALDI-TOF spectrum for young animals receiving copper sulfate (green curve) and its model spectrum (blue curves). B – model spectra for three different cases: experimentally obtained spectrum (MT + nCu), MTs that did not bind copper ions (apo- MT), expected spectrum for pronounced cooperative copper binding (MT + 8Cu).

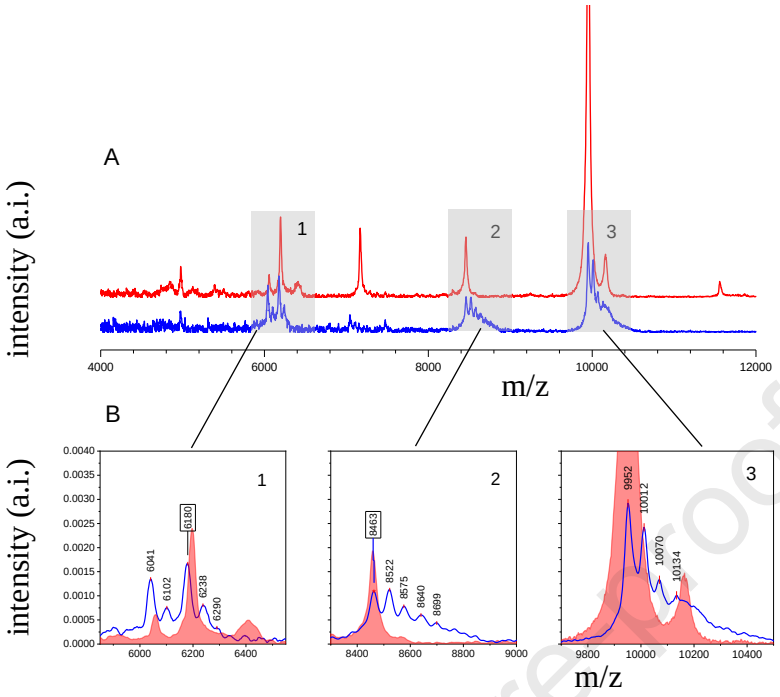
According to the data of the intracellular nature of the distribution of copper ions in the cell compartments, only part of the copper is bound to MTs, the rest of the copper does not enter into a complex with MT, but is bound to other proteins. The data obtained suggest that the mechanism of copper binding in MTs does not correspond to the cooperative model, but to a greater extent corresponds to the model of non-cooperative binding of copper to MTs. It follows from this that copper ions are weakly bound to MTs and can be easily transferred to other proteins.

The reversibility of the binding of copper ions to MTs indicates that MTs are able not only to function as a temporary depot for copper atoms, but also to transfer copper to other proteins, that is, they also perform a transport function, and they are capable of highly dynamic exchange of copper with other ligands.

This is also supported by the data on the formation of complexes of various proteins with copper included in the LPC (and, probably, other proteins) after the administration of $CuSO_4 \cdot 5H_2O$ into the system *in vitro*. For this purpose, $CuSO_4 \cdot 5H_2O$ was additionally introduced into the obtained samples at a concentration of 0.25 mg per 1 ml; the aim was to investigate the binding specificity in vitro by providing copper ion availability to all proteins in the samples.

It turned out that complexes with copper, which were previously observed only for MTs, after addition in the *in vitro* system, were now revealed for almost all proteins that are present in the MALDI-TOF spectra (Fig. 8). Under such conditions, we did not find binding specificity,

that is, MTs do not show high affinity for copper in comparison with other proteins of the liver cytosol, at least in the *in vitro* system.



spectra, after adding copper sulfate to the test samples in vitro general view

detailed view

Fig. 8. Typical spectra after adding $CuSO_4 \cdot 5H_2O$ to low molecular weight proteins of the cytosol in an *in vitro* system for young experimental animals: the red spectrum corresponds to the spectrum in Fig. 6.A.2, and the blue – after adding $CuSO_4 \cdot 5H_2O$ to the LPS samples of the same groups of animals at a concentration of 0.25 mg per 1 ml. A – General view of the spectrum. B – Detailed view of the spectra of copper complexes (1, 2 and 3).

Clearly, it cannot be ruled out that the binding of copper ions to proteins in the *in vitro* system differs from the binding of copper in the cell *in vivo*. However, the results indicate that MTs bind copper ions non-cooperatively, while they are able to bind relatively large amounts of copper (up to 6-8 atoms), in contrast to other cell proteins, as shown in the model (fig. 7.B). But, they “easily” “give” it to other proteins, i.e., they are able to perform a transport function, and not the function of a neutral depot. The results suggest the functioning of the resistance formation mechanism to high doses of copper sulfate - by the mechanism of "functional systemic restructuring" of specific and nonspecific components of the cell, providing copper exchange in the liver and the body as a whole.

4.4. Influence of "excessive" binding of copper ions with components of liver cells on their functional activity.

When considering the toxic effect of heavy metal ions, two mechanisms are most often discussed: 1 – induction of oxidative stress; 2 – complexation with enzymes or other functionally active macromolecules and, as a consequence, suppression of their activity [37; 38]. It turned out that a multiple increase in the content of copper ions in mitochondria was accompanied by a slight increase in the content of lipid hydroperoxides (HPL) in mitochondria in young and old animals by 29-35%, respectively (Fig. 9.A).

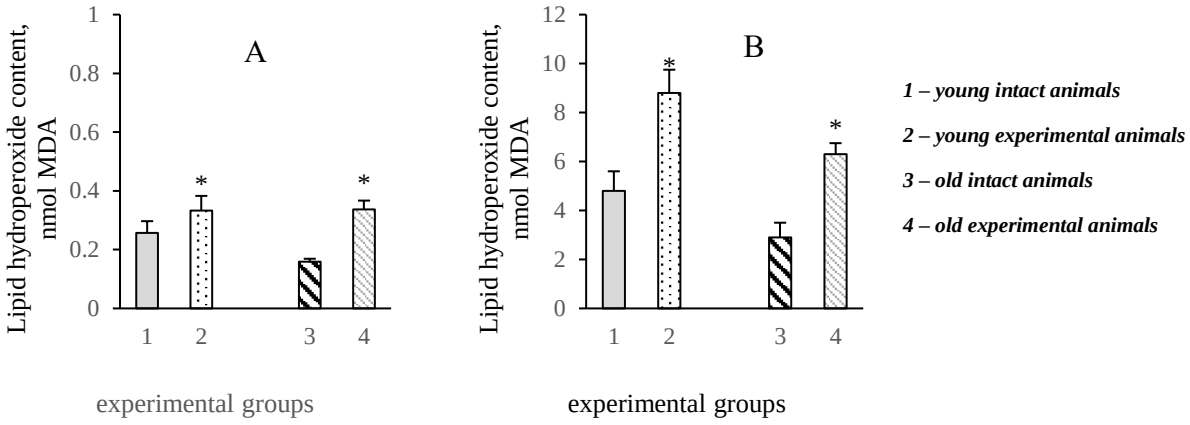


Fig. 9. Content of HPL in mitochondria (A) and blood serum (B) in intact young animals (1), young animals after three administration of copper sulfate (2) and, accordingly, in intact old animals (3) and old animals after three consecutive administrations copper sulfate (4) (there were 6 animals in each group). * – P<0.05 between young/old animals receiving triple copper sulfate in relation to their intact control.

The content of HPL was also increased in the blood serum of young and old animals by 102% and 90%, respectively (Fig. 9.B). This indicates in animals that were administrated three times with copper sulfate, the equilibrium in the prooxidant-antioxidant system was the body is biased towards prooxidants and depends little on age.

As is known, the content of the products of free radical reactions depends on the relationship between the induction of reactive oxygen species by copper ions and the activity of the antioxidant system [39]. In the next series of experiments, the activity of the key enzyme of the antioxidant defense – glutathione peroxidase – was determined in mitochondria, which contained a large amount of copper ions. We found that copper ions inhibit its activity by only 15-25%, respectively, in young and old animals. This change correlated with an increase in the content of hydroperoxides in mitochondria (Fig. 10.A).

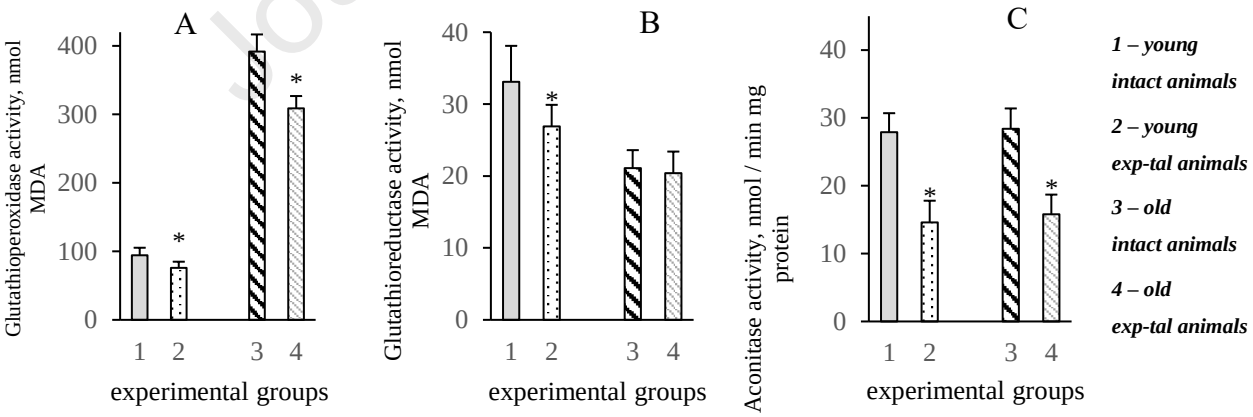


Fig. 10. Activity of glutathione peroxidase (A), glutathione reductase (B) and aconitase (C) in liver mitochondria in intact young animals (1), young animals after three administration of copper sulfate (2) and, accordingly, in intact old animals (3) and old animals after three consecutive administration of copper sulfate (4) (there were 6 animals in each group), * – P<0.05 between young/old animals receiving triple copper sulfate in relation to their intact control.

It is known that glutathione reductase is another enzyme of the glutathione cycle. Its activity in mitochondria "loaded" with copper ions was slightly reduced in young animals by 30%, while in the mitochondria of old animals it did not differ from the control level (Fig. 10.B).

The most important indicator of the functional activity of mitochondria is the activity of aconitase. In our investigation, an increase (16 times) in the content of copper ions in mitochondria compared to the control level was accompanied by a loss of aconitase activity in mitochondria only by 47.7% and 44.4% compared to control, in young and old animals, respectively (Fig. 10. C). A decrease in aconitase activity in mitochondria correlated with an increase in the content of HPL in the body.

The results obtained allow us to conclude that sequential threefold administration of adaptive doses (0.23 mg / 1 g of liver) of copper sulfate, which was accompanied by a multiple increase in the content of copper ions in the ER and mitochondria, slightly increased the content of hydroperoxides, which correlated with inhibition of glutathione peroxidase and aconitase, and this did not depended on the age of the animals. Consequently, despite the multiple increases in the content of copper ions in the compartments of the liver cell, with the sequential administration of adaptive doses of copper sulfate, the functional activity of the enzymes changed insignificantly, and oxidative stress is manifested to a small extent compared to the increase in the content of copper ions. Subsequent administration of lethal doses of copper sulfate to such animals did not lead to their death. This suggests that the mechanism of induced resistance to lethal doses is realized at different hierarchical levels, at least at the level of induction of stress proteins, and at the level of molecular acclimation, i.e. the formation of "resistance" of proteins to any negative environmental factor, in this case to high concentrations of copper ions.

5. Discussion

The results of the study allow us to conclude:

1 – the sequential administration of copper sulfate small doses (about 30% of the lethal dose) ensured the resistance formation to subsequent lethal doses of copper sulfate. The old ones differed from the young ones in the temporal characteristics of the adaptive process, which may indicate that animals of different ages use different mechanisms of the formation of resistance to toxicants;

2 – in liver cells of intact young and old animals, more than 80% of copper ions are associated with MTs. The MTs content in the liver cytosol in old animals before the administration of copper sulfate was higher than in young animals, i.e. the baseline in the old was different from the baseline in the young;

3 – three-fold sequential administration of small adaptive doses of copper sulfate to animals was accompanied by an increase in MTs content in liver cells of young animals and, to a lesser extent, in cells of old animals. At the same time, the content of copper ions bound to MTs decreased and amounted to about 60% of all copper, which bound to mitochondrial proteins, ER, and cytosolic proteins, that is, the content of copper ions bound to other ligands is increased;

4 – copper ions bind to MTs non-cooperatively and, probably, are easily redistributed over the cell compartments; MTs provide transport of copper in the cell. As is known, the molecules of MTs are characterized by a high dynamism of the secondary structure (double folding), and the tertiary structure consists of two clusters and, due to the high content of cysteine, they bind Cu and Zn ions. These bonds are characterized by a large variability of the energy of coordination bonds, and different metals are characterized by different affinities to MTs (Ag> Hg> Cu> Gl> Zn> Co) [40]. This feature of the binding of metal ions, in particular copper, provides a high bioavailability of the metal, which is confirmed by the results of this work. For Zn ions, the possibility of transfer from albumin to MTs and further to other proteins, which have a larger binding constant, has been shown [41];

5 – an increase in the content of copper ions by tens of times in the fractions of mitochondria and ER was accompanied by a slight inhibition of antioxidant enzymes and an increase in the products of free radical reactions. This can be considered as a regulatory mechanism for adaptation to the action of a toxicant, that is, in the process of adaptation, the

binding capacity of proteins in relation to copper increased without a proportional inhibition of their activity;

6 – a study of the long-term effects of the copper sulfate action, i.e. the administration of copper sulfate in two series with an interval between injections of one month, showed the presence of an age-dependent nature of the intracellular distribution of copper ions and a significant increase in the degree of copper binding to MTs; in old animals and to other cell compartments while maintaining their main functional activity. This suggests the presence of a mechanism of molecular acclimation (adaptation of protein molecules to one of the environmental factors, in this case to an excess amount of copper ions).

The results obtained confirm the hypothesis of a hierarchical mechanism of induced resistance to high doses of copper sulfate, which is realized in the liver. The appearance of copper ions in the liver is accompanied by the induction of MTs synthesis, which are capable of binding up to 6-8 copper atoms. In this case, copper ions also bind to other proteins of all cell compartments. MTs as small molecules (6 kDa) are capable of highly dynamic movements in the cell; they easily transfer copper to other structures, i.e. there is a high dynamics of copper in the "ping-pong principle" cage; they attach for a short time and are quickly cleaved, while maintaining the functional activity of many enzymes. It can be assumed that as a result of such “ping-pong” of copper ions, many proteins are capable of acclimation, and after repeated cycles of encountering these negative factors, they are able to increase the binding of additional copper ions while maintaining their functional activity.

The ability of a number of proteins to bind a relatively large number of copper ions without significant changes in their structure is evidenced by the data of infrared spectrophotometry of copper-binding proteins [42].

Consequently, various elements are involved in the formation of resistance to copper ions excess, forming different adaptation levels (Fig. 11).

The available data suggest that, at the level of liver functional characteristics, in response to the administration of copper sulfate large doses into the body, old animals use a slightly different combination (share) of elements providing the mechanism of induced resistance or another strategy of an adaptive response. The choice of the adaptation strategy to high concentrations of copper ions is more influenced by the initial level of the metabolic system, and it was different in young and old animals (Fig. 11).

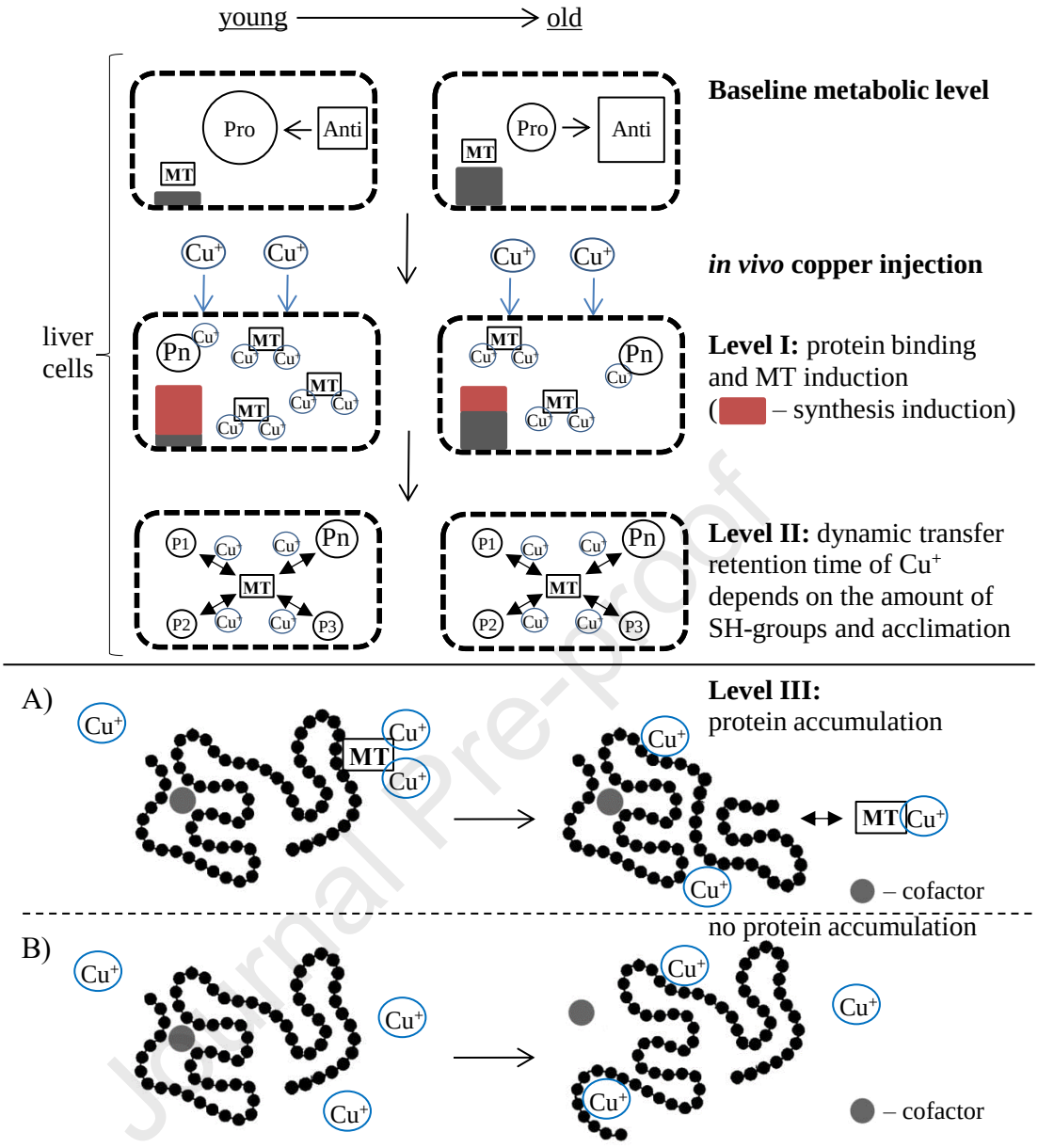


Fig. 11. The scheme demonstrates the difference between the initial level in the content of metallothioneins (MTs are marked as histogram) and the characteristics of the redox system (prooxidants - antioxidants). In young animals, the balance is shifted towards prooxidants, and in old ones, towards antioxidants. The administration of copper ions into the body leads to the binding of these ions with various groups of proteins in liver cells, in particular with MTs, ER, cytosolic proteins, which are conventionally noted as P1, P2, P3 ... Pn, and induces MTs synthesis in varying degrees in young and old animals (histogram) – level I. MTs provide dynamic transfer of copper ions between different proteins according to the “ping-pong” principle – level II. As a result of such "bombardment" of proteins by copper ions, some of them are capable of acclimation to copper ions, can retain (unchanged active center) and bind an increased amount of copper.

It can be assumed that small changes in individual elements of regulatory systems are capable of exhibiting a synergistic effect, which can lead to the emergence of new properties of the system, in contrast to the properties of individual elements of the system, and give a greater quantitative effect than a simple sum, which is manifested in an increased effect of survival on physiological level, against the background of "small" biochemical changes.

The choice of one or another adaptation strategy in biological systems is carried out in compliance with the principle of optimality, i.e., the response of the system is formed from the available functional elements at a given time or the principle of the initial functional state. It was found that in intact old animals before the administration of copper sulfate, the liver contained significantly more MTs in comparison with young animals (Fig. 6.A), which coincided with an increase in the survival of old animals after the first injection of copper sulfate large doses (Fig. 2.A). However, after threefold administration of adaptive doses of copper sulfate, MTs induction in old rats was less pronounced than in young ones. This coincided with an increase in the percentage of surviving young animals after the administration of adaptive doses of copper sulfate, compared with old animals (Fig. 2.C).

As is known, MTs are polyfunctional proteins, and their induction is carried out not only by heavy metal ions, but also by a number of other negative factors; therefore, they are referred to the family of stress proteins [43]. It can be assumed that during its relatively long ontogenesis, the organism of old animals has already met with various inducers of stress proteins, including MTs. Consequently, the initial states against which the adaptive strategy to copper ions is realized were different in young and old animals. Young and old animals differed in the characteristics of the initial states and in relation to the activity of antioxidant enzymes (glutathione peroxidase and glutathione reductase), and in the content of lipid hydroperoxides (Fig. 9, 10) and, probably, in other metabolic systems.

The most important aspect of the functioning of the principle of optimality is the time of preservation of previously induced metabolic patterns, i.e. the presence of metabolic memory [44]. As shown, after the first series of triple consecutive injections of copper sulfate, LPC bound about 5-6 μg of copper per 1 mg of protein, and one month after the complete release of these proteins from copper ions, they were able to bind up to 15 μg, and in old animals even up to 30 μg copper per mg protein, i.e. it can indirectly confirm the ability of proteins to acclimate. These data indicate that induced copper-binding proteins are able to "persist" even after the effect of the inducer is removed, i.e. memorized, and this will influence the choice of a new adaptation strategy based on the principle of optimality.

The most important aspect in the mechanism of induced resistance to high doses of copper sulfate is the possible molecular adaptation of protein molecules, or rather acclimation, i.e. adaptation of specific types of molecules to one experimental factor; in this case, to high doses of copper ions. Several facts can indirectly testify in favor of molecular acclimation of proteins: firstly, after preliminary injections of small doses of copper sulfate in animals, resistance to subsequent injections of a lethal dose is formed, and the intracellular pattern of distribution of copper ions changes; secondly, a multiple increase in the content of copper ions in mitochondria and ER do not affect or insignificantly affect the activity of a number of enzymes that make up these structures; thirdly, after repeated cycles of the administration of copper sulfate, LPC increased the binding capacity, i.e. “has learned” to bind many times more copper ions while preserving their functions.

In conclusion, we note that with increasing age, the ability to adapt to the excess content of copper ions in the body does not decrease, but the adaptation strategy changes, i.e. combinatorics of elements involved in the formation of resistance. A possible change in adaptation strategies with age was shown on a model of a regenerating liver [45], and the development of an infectious process [46]. The choice of adaptive strategies is based on the principle of metabolic optimality, which in turn depends on the initial functional state of systems, metabolic memory, and molecular acclimation, the interaction of which determines emergence.

5. Conclusions

1. Multiple preliminary administrations of small doses of copper sulfate induce the resistance formation to subsequent lethal doses of this toxicant.

2. The age-dependent nature of MTs induction and a change in the nature of the copper ions distribution in liver cells is observed against the background of induced resistance.

3. MTs are involved in the protein transfer of copper ions and this process is age-dependent in animals.

References

1. Baldo, B. A., Rose, M. A. (2020). The anaesthetist, opioid analgesic drugs, and serotonin toxicity: a mechanistic and clinical review. *British journal of anaesthesia*, *124*(1), 44-62. https://doi.org/10.1016/j.bja.2019.08.010.

2. Cong, Y., Han, X., Wang, Y., Chen, Z., Lu, Y., Liu, T., Wu, Z., Jin, Y., Luo, Y., Zhang, X. (2020). Drug toxicity evaluation based on organ-on-a-chip technology: A review. *Micromachines*, *11*(4), 381. https://doi.org/10.3390/mi11040381.

3. Sabe, M., Dorsaz, O., Huguelet, P., Kaiser, S. (2021). Toxicity of psychotropic drugs in patients with COVID-19: A systematic review. *General hospital psychiatry*, 70, 1-9.3. https://doi.org/10.1016/j.genhosppsych.2021.02.006.

4. Aldossary, S. A. (2019). Review on pharmacology of cisplatin: clinical use, toxicity and mechanism of resistance of cisplatin. *Biomedical and Pharmacology Journal*, *12*(1), 7-15. https://dx.doi.org/10.13005/bpj/1608.

5. Vardhan, K. H., Kumar, P. S., & Panda, R. C. (2019). A review on heavy metal pollution, toxicity and remedial measures: Current trends and future perspectives. *Journal of Molecular Liquids*, *290*, 111197. https://doi.org/10.1016/j.molliq.2019.111197.

6. Gupta, N., Yadav, K. K., Kumar, V., Kumar, S., Chadd, R. P., & Kumar, A. (2019). Trace elements in soil-vegetables interface: translocation, bioaccumulation, toxicity and amelioration-a review. *Science of the Total Environment*, *651*, 2927-2942. https://doi.org/10.1016/j.scitotenv.2018.10.047.

7. Flemming, C.A., Trevors, J.T., 1989. Copper toxicity and chemistry in the environment: a review. *Water, Air, and Soil Pollution* 44 (1-2), 143-158. https://doi.org/10.1007/BF00228784.

8. Yruela, I., 2009. Copper in plants: acquisition, transport and interactions. Functional Plant Biology 36 (5), 409-430. https://doi.org/10.1071/FP08288.

9. Jensen, E.L., Gonzalez-Ibanez, A.M., Mendoza, P., Ruiz, L.M., Riedel, C.A., Simon, F., et al., 2019. Copper deficiency-induced anemia is caused by a mitochondrial metabolic reprograming in erythropoietic cells. *Metallomics* 11 (2), 282-290. https://doi.org/10.1039/C8MT00224J.

10. Ingle, A.P., Paralikar, P., Shende, S., Gupta, I., Biswas, J.K., da Silva Martins, L.H., Rai, M., 2018. Copper in Medicine: Perspectives and Toxicity. *In Biomedical Applications of Metals*, 95-112. https://doi.org/10.1007/978-3-319-74814-6_4.

11. Zatulovskaia, Y.A., Ilyechova, E.Y., Puchkova, L.V., 2015. The features of copper metabolism in the rat liver during development. *PloS one* 10 (10), e0140797. https://doi.org/10.1371/journal.pone.0140797.

12. Zoroddu, M.A., Aaseth, J., Crisponi, G., Medici, S., Peana, M., Nurchi, V.M., 2019. The essential metals for humans: a brief overview. *Journal of inorganic biochemistry* 195, 120-129. https://doi.org/10.1016/j.jinorgbio.2019.03.013.

13. Ebara, M., Fukuda, H., Hatano, R., Saisho, H., Nagato, Y., Suzuki, K., et al., 2000. Relationship between copper, zinc and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma. *Journal of hepatology* 33 (3), 415-422. https://doi.org/10.1016/S0168-8278(00)80277-9.

14. Bozhkov, A.I., Sidorov, V.I., Kurguzova, N.I., Dlubovskaya, V.L. (2014). Metabolic memory enhances the effect of hormesis to copper ions and is age-dependent. *Advances in Gerontology*, 27 (13), 72-80.

15. Rathbun, J.K., 1996. Neuropsychological aspects of Wilson's disease. International *Journal of Neuroscience* 85 (3-4), 221-229. https://doi.org/10.3109/00207459608986684.

16. Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M.A., Alvi, R.F., Aslam, M.A., Qamar, M.U., Salamat, M.K.F., Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance*, *11*, 1645. https://doi.org/10.2147/IDR.S173867.

17. Jayawardena, D.P., Heinemann, I.U., Stillman, M.J., 2017. Zinc binds non-cooperatively to human liver metallothionein 2a at physiological pH. B*iochemical and biophysical research communications* 493 (1), 650-653. https://doi.org/10.1016/j.bbrc.2017.08.137.

18. Krężel, A., Maret, W., 2017. The functions of metamorphic metallothioneins in zinc and copper metabolism. *Int. J. Mol. Sci.* 18, 1237. https://doi.org/10.3390/ijms18061237.

19. Freisinger, E., Vasak, M., 2013. Cadmium in metallothioneins. *Met. Ions Life Sci*. 11, 339–371.

20. Marcelo, G. A., Font, A., & Gallego, A. (2017, July). Antioxidants and chaperones gene expression in immune cells of Antarctic sea urchin by stress inductors. In *Book of Abstracts* (p. 310).

21. Harmaza, Y. M., Tamashevski, A. V., & Slobozhanina, E. I. (2019). The role of metallothioneins in maintenance of zinc homeostasis and redox state in erythrocytes of cardiologic patients with metabolic syndrome. *Journal of Integrated OMICS*, *9*(1), 10-16. https://doi.org/10.5584/jiomics.v9i1.260.

22. Bozhkov, A. I., Nikitchenko, Y. V., Klimova, E. M., Linkevych, O. S., Lebid, K. M., Al-Bahadli, A. M. M., Alsardia, M. M. A. (2017). Young and old rats have different strategies of metabolic adaptation to Cu-induced liver fibrosis. *Advances in Gerontology*, *7*(1), 41-50. https://doi.org/10.1134/S2079057017010040.

23. Bozhkov, A. I., Ivanov, E. G., Kuznetsova, Y. A., Ohiienko, S. L., & Bondar, A. Y. (2017). Copper-induced liver fibrosis affects the behavior of bone marrow cells in primary culture. *Frontiers in biology*, *12*(4), 271-279.

24. Bozhkov, A. I., Klimova, O. M., Nikitchenko, Y. V., Kurguzova, N. I., Linkevych, O. S., Lebid, K. M., Protsenko, O.S., Remneva, N.A., Al-Bahadly, A.M.M., Al-Begai, M. A. (2017). Ontogenetic approach to the study of mechanisms of copper-induced liver fibrosis. *Advances in Aging Research*, *6*(03), 39. https://doi.org/10.1007/s11515-017-1458-y.

25. Bozhkov, A. I., Ohiienko, S. L., Bondar, A. Y., Klimova, E. M., & Ivanov, E. G. (2019). Induced Liver Fibrosis Is Accompanied in Young and Old Animals by Age-Dependent Changes in Bone Marrow Cells. *Advances in Gerontology*, *9*(3), 289-297. https://doi.org/10.1134/S2079057019030032.

26. Klimova, E. M., Bozhkov, A. I., Kovalenko, T. I., Minukhin, V. V., Belozerov, I. V. (2018). Young and old animals use different strategies for forming an immune response to infectious agents (Pseudomonas aeruginosa and Escherichia coli). *Advances in Gerontology*, *8*(4), 284-291. https://doi.org/10.1134/S2079057018040082.

27. Bozhkov, A., Padalko, V., Dlubovskaya, V., Menzianova, N., 2010. Resistance to heavy metal toxicity in organisms under chronic exposure. *Indian Journal of Experimental Biology* 48, 679-696 Jul.

28. Kaplan, E.L., Meier, P., 1958. Nonparametric estimation from incomplete observations. *Journal of the American statistical association* 53 (282), 457-481. https://doi.org/10.1080/01621459.1958.10501452.

29. Kamath, S.A., Narayan, K.A., 1972. Interaction of Ca2+ with endoplasmic reticulum of rat liver: a standardized procedure for the isolation of rat liver microsomes. *Analytical Biochemistry* 48(1), 53-61. https://doi.org/10.1016/0003-2697(72)90169-8.

30. Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical biochemistry* 95 (2), 351-358. https://doi.org/10.1016/0003-2697(79)90738-3.

31. Asakawa, T., Matsushita, S., 1980. Coloring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids* 15 (3), 137-140. https://doi.org/10.1007/BF02540959.

762 32. Massie, H.R., Aiello, V.R., Banziger, V., 1983. Iron accumulation and lipid
763 peroxidation in aging C57BL/6J mice. *Experimental gerontology* 18(4), 277-285.
764 https://doi.org/10.1016/0531-5565(83)90038-4.
765 33. Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative
766 characterization of erythrocyte glutathione peroxidase. T*he Journal of laboratory and clinical*
767 *medicine* 70 (1), 158-169.
768 34. Carberg, I., Mannerviek, B., 1975. Glutathione reductase levels in rat brain. *J. Biol.*
769 *Chem.* 250, 5475–5480.
770 35. Varghese, S., Tang, Y., Imlay, J.A., 2003. Contrasting sensitivities of Escherichia coli
771 aconitases A and B to oxidation and iron depletion. *Journal of bacteriology* 185 (1), 221-230.
772 https://doi.org/10.1128/JB.185.1.221-230.2003.
773 36. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement
774 with the Folin phenol reagent. *J. Biol. Chem*. 193 (1), 265-275 Nov.
775 37. Sato, M., Bremner, I., 1993. Oxygen free radicals and metallothionein. *Free Radical*
776 *Biology and Medicine* 14 (3), 325-337. https://doi.org/10.1016/0891-5849(93)90029-T.
777 38. Bozhkov, A.I., Nikitchenko, Y.V., Lebid, K.M., Ivanov, E.G., Kurguzova, N.I.,
778 Gayevoy, S.S., Sharko, M.O., Alsardia, M.M.A., Al-Begai, M.A.Y., 2017. Low molecular
779 weight components from various sources eliminate oxidative stress and restore physiological
780 characteristic of animals at early stages of Cu-induced liver fibrosis development. *Translational*
781 *Biomedicine* 8 (2), 107.
782 39. Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.L., Lesnefsky, E.J., 2003.
783 Production of reactive oxygen species by mitochondria central role of complex III. *Journal of*
784 *Biological Chemistry* 278 (38), 36027-36031. https://doi.org/10.1074/jbc.M304854200.
785 40. Waalkes, M.P., Harvey, M.J., & Klaassen, C.D., 1984. Relative *in vitro* affinity of
786 hepatic metallothionein for metals. *Toxicology letters* 20 (1), 33-39.
787 https://doi.org/10.1016/0378-4274(84)90179-6.
788 41. Quiming, N.S., Vergel, R.B., Nicolas, M.G., & Villanueva, J.A., 2005. Interaction of
789 bovine serum albumin and metallothionein. *Journal of health science* 51 (1), 8-15.
790 https://doi.org/10.1248/jhs.51.8.
791 42. Bozhkov, A.I., Sidorov, V.I., Dlubovskaya, V.L., Shevtsova, M.Y., 2010. Appearance
792 of the imprinting effect on the specific pattern of intracellular distribution of copper ions in the
793 liver after exposure to high concentrations of copper sulfate. *Biomeditsinskaya khimiya* 56 (2),
794 195-208. https://doi.org/10.18097/pbmc20105602195.
795 43. Theocharis, S.E., Margeli, A.P., Koutselinis, A., 2003. Metallothionein: a
796 multifunctional protein from toxicity to cancer. The International journal of biological markers
797 18 (3), 162-169. https://doi.org/10.1177/172460080301800302.
798 44. Bozhkov, A.I., Nikitchenko, Y.V., Al-Bahadly, A.M.M., 2016. Overeating in Early
799 Postnatal Ontogenesis Forms Metabolic Memory and Reduces Lifespan. J. Gerontol. Geriatr.
800 Res. 5 (309), 1-9. https://doi.org/10.4172/2167-7182.1000309.
801 45. Palmes, D., Spiegel, H.U., 2004. Animal models of liver regeneration. Biomaterials
802 25 (9), 1601-1611. https://doi.org/10.1016/S0142-9612(03)00508-8.
803 46. Kak, V., Sundareshan, V., Modi, J., Khardori, N.M., 2012. Immunotherapies in
804 infectious diseases. Medical Clinics 96 (3), 455-474. https://doi.org/10.1016/j.mcna.2012.04.002.
805