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Alterations in the expression of TfR1, DMT-1 and Hepcidin in immature mice liver after chronic exposure to cobalt chloride

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Cobalt (Co) is an essential trace element and its cellular uptake follows a similar pattern to that of iron (Fe). Our aim was to study the effects of perinatal cobalt chloride ($CoCl_2$) exposure on iron homeostasis through changes in the expression of iron regulatorty proteins - transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT-1) and hepcidin (Hepc) in the liver of 30-day old mice. Pregnant mice and their progeny were treated with daily doses of 75 mg/kg b.w. (low dose) or 125 mg/kg b. w. (high dose) $CoCl_2$ until postnatal day 30. The results show that prenatal and early postnatal exposure to $CoCl_2$ exerts a toxic effect on the liver, reducing the organ weight index and altering the expression profile of the proteins involved in iron metabolism regulation. Weaker expression of TfR1 and DMT-1 was observed in the samples of mice exposed to the low dose $CoCl_2$.

Key words: cobalt chloride, iron-regulatory proteins, liver, in vivo model, toxicity

Introduction

Cobalt (Co) is an essential trace element for human and animals, but high doses can negatively affect human health. The average daily intake of Co ranges from 5 to 45 μg, with food sources such as leafy vegetables, meat, fish, dairy products, and drinking water being the main contributors of cobalt in the general population's diet. Organic Co plays a key role as a metal component in vitamin B12 (cyanocobalamin), while inorganic cobalt compounds are considered toxic to both the environment and humans when present to excessive levels [4]. Studies on long-term exposure of laboratory animals to Co ions show that they accumulate in organs such as kidney, liver, spleen, heart, stomach, intestines, muscle, brain and testis [6]. The concentration of Co is also elevated in blood, serum and urine [14]. If cobalt concentration exceeds normal levels in the body, its ions compete with iron (Fe) for

binding to macromolecules, disrupting the activity of iron-regulatory proteins and altering various biochemical pathways [5].

The existing data suggest that Co is taken up by cells through transferrin receptors interaction, with an uptake pattern similar to that of iron. The serum iron transport protein- transferrin (Tf) can carry not only two Fe^{3+}, but also other metal ions, such as cobalt. Differic Tf is bound to membrane TfR1 and then internalized by clathrin-dependent endocytosis. Iron, following its reduction to Fe^{2+} by STEAP3, is then exported to the cytosol via divalent metal transporter 1 (DMT-1), and TfR1 is recycled back to the cell surface [18]. Iron is either used in metabolic processes, such as the synthesis of hemoproteins and Fe-S clusters, transferred to the labile iron pool, or exported from the cell by ferroportin (FPN) [2]. Iron metabolism is regulated through the hepcidin/ ferroportin axis at the systemic level, with hepcidin acting as a negative regulator of iron flow.

There are limited data on the *in vivo* effects of Co exposure on tissue-specific expression of proteins involved in iron metabolism (TfR1, DMT1, Hepc) and their complex interactions, along with the associated health risks. The aim of the present study is to investigate the alterations in Fe- regulatory proteins (TfR1, DMT-1, hepcidin) expression in the liver of immature (30-day-old) mice after chronic exposure to cobalt chloride.

Materials and Methods

I. Experimental design

Experimental animals were purchased from the Experimental and breeding base for laboratory animals (EBBLA) – Slivnitza, Bulgaria. After one-week acclimatization female ICR (Institute of Cancer Research) mice were mated to male ICR mice. Once pregnancy was confirmed, the female ICR mice were placed in individual standard polypropylene cages and treated 2-3 days before delivery with cobalt chloride ($CoCl_2 \times 6H_2O$) at a daily dose of 75 mg/kg b.w. (low dose) or 125 mg/kg b.w. (high dose) until the offspring reach 30 days of age. $CoCl_2$ was dissolved in tap water to ensure that mice obtained the required daily dose. The mothers were provided with 15 ml per day and the 25-day-old mice received 3 ml of the solution. Mice were kept in the institute's animal facility at a temperature of 23 ± 2 °C, with a 12:12h light- dark cycle. The weight changes of the experimental mice were monitored weekly to adjust the dosage accordingly. Additionally, the daily consumption of the test solution or tap water was monitored. On postnatal day 25 the pups were housed in individual cages to ensure equal access to the solution. The treatment continued until postnatal day 30. Age-matched animals obtaining regular tap water, served as controls. The newborn pups were sacrificed on day 30, and their livers were excised and prepared for future analysis. No significant differences were observed in the indicators between male and female experimental animals, which is why both sexes are included in the present study.

The experimental design was carried out in accordance with to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and EU Directive 2010/63/EU for animal experiments. The study was approved by the Bulgarian Agency for Food Safety, Approval number 282 from 24.09.2020.

II. Morphological studies

Liver's tissue samples were fixed in Bouin solution for 24 hours, dehydrated in an ascending alcohol series up to 100%, cleared with xylene, impregnated in molten paraffin and then embedded in fresh molten paraffin. The tissues were cut to 5 μm thick sections, which were later used for immunohistochemical studies.

II.a. Liver index

Livers of controls and Co-treated mice were excised, weighed and liver index was calculated as a ratio of liver weight to body weight.

II.b Immunohistochemistry for TfR1, hepcidin and DMT-1

All incubations were conducted at room temperature, unless stated otherwise. Initially, the 5μm sections of control and Co-treated animals were deparaffinated and rehydrated, then heated in 0.01M citrate buffer (pH 6.0) for antigen retrieval. In the case of DMT1, a 0.05M glycine buffer (pH 3.5 and 0.01% EDTA) was used. Endogenous peroxidase activity was blocked with 3% H_2O_2 dissolved in methanol, followed by four 5-minute washes in TBS. To block nonspecific background staining, the sections were incubated for 5 minutes with Super Block. After the 5-minute washing steps were repeated, a blocking kit containing avidin and biotin (Vector Laboratories, Inc., CA, USA) was added to block endogenous biotin. Primary rabbit polyclonal anti-TfR1 antibody, anti- hepcidin antibody and anti-DMT1 antibody were added (for TfR1- 1:100, for hepcidin- 1:400, for DMT1- 1:100; Wuhan Elabscience Biotechnology Co. Ltd., China) and the samples were incubated overnight at 4°C. After four 5-minute washes in TBS, sections were incubated with a biotinylated secondary antibody. After four additional 5-minute washes in TBS, sections were incubated with anti-rabbit UltraTek HRP for 10 min (UltraTek HRP Anti-Mouse Staining System, ScyTek Laboratories, USA). Sections were washed four times in TBS, and the immunohistochemical reaction was visualized with 3,3'- diaminobenzidine (liquid DAB; DAKO Corp.). The reaction was stopped with water and then the sections were counterstained with hematoxylin and mounted in Entallan. The sections were observed under a Leica DM 5000B light microscope (Leica Microsystems, USA).

Negative controls were represented by samples incubated without the primary antibody.

III. Statistical analysis

The results are presented as mean values ± Standart Deviation (SD). The significance of the differences between the experimental groups was assessed using one-way ANOVA with Bonferroni post-hoc test at a significance level of $p<0.05$.

Results and Discussion

Previous studies show that cobalt is transferred from food into the mother's milk [10], which means that the newborn pups were exposed to Co during their nursing period. The body weight of Co-exposed animals was affected by chronic treatment with $CoCl_2$, indicating potential changes in liver weight index as well.

Prenatal and early postnatal exposure to $CoCl_2$ resulted in a decreased liver index, with the reduction being significant ($p \leq 0.001$) in the mice exposed to the higher dose of $CoCl_2$ (125 mg/kg b.w.) **(Fig. 1.)**. Hepatocytes are crucial targets in both acute and chronic toxicity. Subsequently, the liver index is an important indicator in toxicological studies. It reflects the impact of various hepatotoxins, and its decrease is usually associated with a reduction in functional activity and/or hepatocellular damage [1]. The significant reduction in the liver index after chronic $CoCl_2$ intake demonstrates its toxic effects in 30-day-old immature mice. A lower liver index has also been observed when the experimental animals were treated with other metals, such as cadmium and lead [20]. The liver is responsible for synthesis of key factors in iron metabolism; therefore, changes in liver index will lead to alterations in Fe content. Our previous data revealed significantly increased iron levels in the liver tissue following exposure to $CoCl_2$ [9].

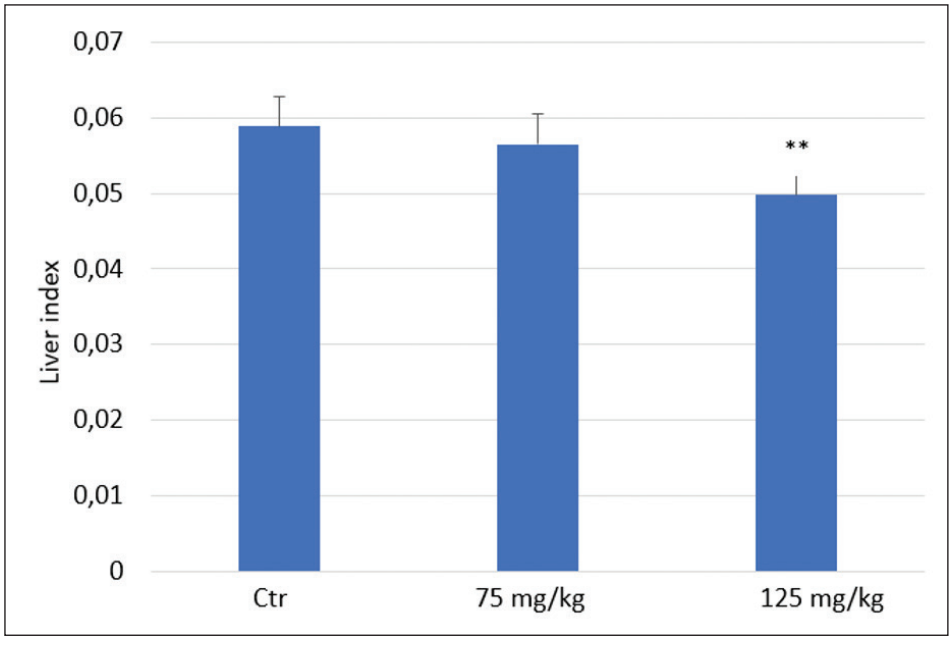

Fig. 1. Changes in liver index (liver weight to body weight ratio) of day 30 control and Co-exposed mice. **p<0.01

Changes in the expression of the Fe-regulatory proteins TfR1, hepcidin and DMT-1 were observed by immunohistochemistry of liver sections from both control and Co- treated mice. TfR1 is ubiquitously expressed and post-trascriptionally regulated by iron status via the iron regulatory protein system, leading to an increase in TfR1 under low iron conditions and a decrease TfR1 under high iron conditions [3]. We found that TfR1 expression in cobalt-treated 30-day-old mice is primarily detected in Kupffer cells, whereas TfR1 expression in control samples is periportal. When treated with high dose of $CoCl_2$ (125 mg/kg b.w.), receptor expression is not only found in Kupffer cells, but is also periportal **(Fig. 2)** indicating altered pattern of TfR1 distribution in the

liver tissue. Expression of TfR1 is known to be upregulated by hypoxia and $CoCl_2$ is the most commonly used hypoxia-mimicking agent in experimental studies [11]. Our novel data for altered pattern of TfR1 protein expression could be due to cobalt-induced hypoxia rather than iron concentration. The previous scientific data showed that iron accumulation gradient decreased towards the central part of the lobule (periportal). In cases of iron overload, this gradient is even more pronounced [7]. Our data for pronounced expression of TfR1 in Co-exposed mice with a high daily dose of $CoCl_2$ could explain increased iron concentrations in the liver of treated mice. On the other hand, it has been proven that transferrin receptors also bind cobalt, as the mechanism of interaction and accumulation in cells is similar to that of iron [15].

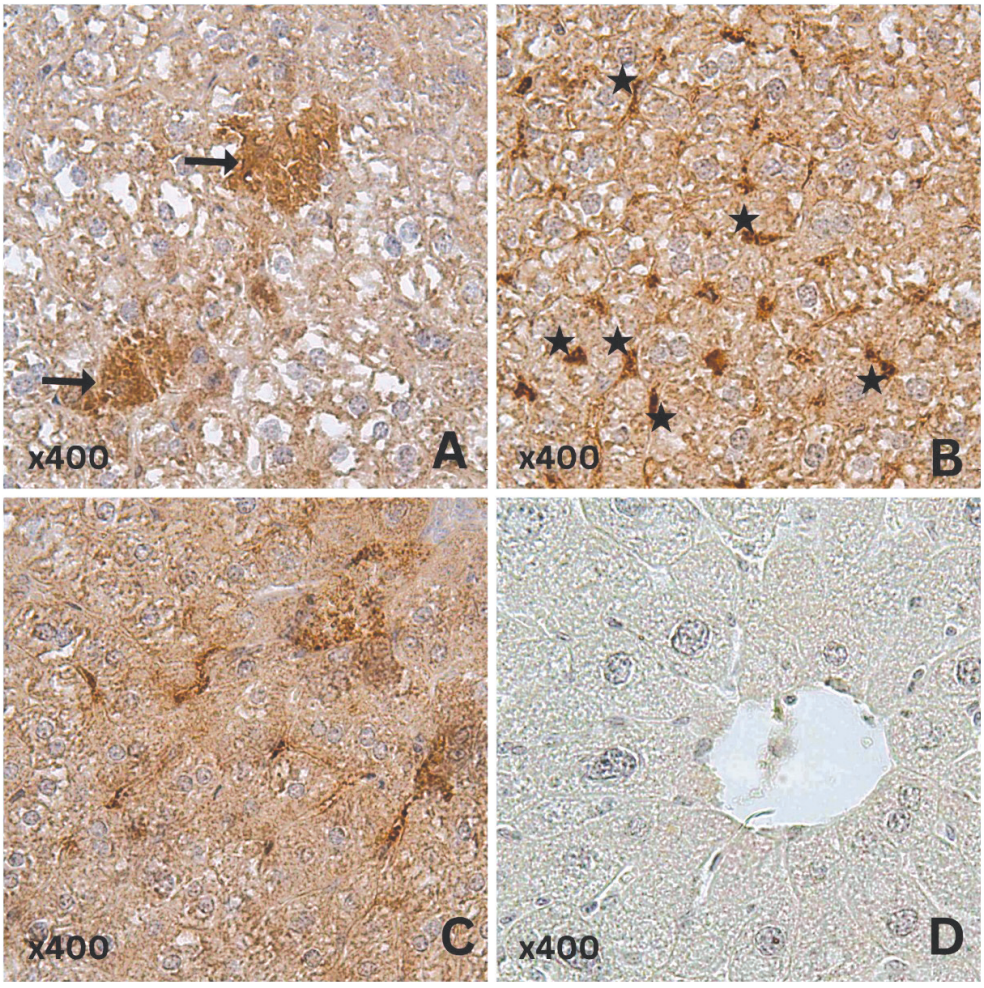

Fig. 2. Immunohistochemical expression of TfR1 in immature mouse liver: Control **(A)**, 75 mg/kg $CoCl_2$ **(B)**, 125 mg/kg $CoCl_2$ **(C)**, negative control **(D)**. TfR1 immunoreactivity in the periportal (arrows) and Kupffer cells (stars). ×400

Our new findings showed that Hepcidin is weakly visualized in the cytoplasm of hepatocytes in the liver of controls and is slightly increased in the $CoCl_2$ treated experimental groups **(Fig.3)**. Hepcidin has a key role to systemic iron regulation, which means that any changes in its expression are of critical importance. Studies show that hepcidin is highly expressed in the liver and has also been detected in various other tissues, including the heart, adipose tissue, alveolar and splenic macrophages, the retina, and different regions of the brain [13]. Hepcidin expression is regulated by body iron levels and rises when iron concentration is high. According to the scientific literature, cobalt-induced hypoxia suppresses hepcidin expression. On the other hand, it has been established that activation of Kupffer cells inhibits hepcidin expression [17]. Furthermore, the weaker hepcidin expression after $CoCl_2$ exposure might stimulate erythropoiesis, which is known to reduce hepcidin concentration [12].

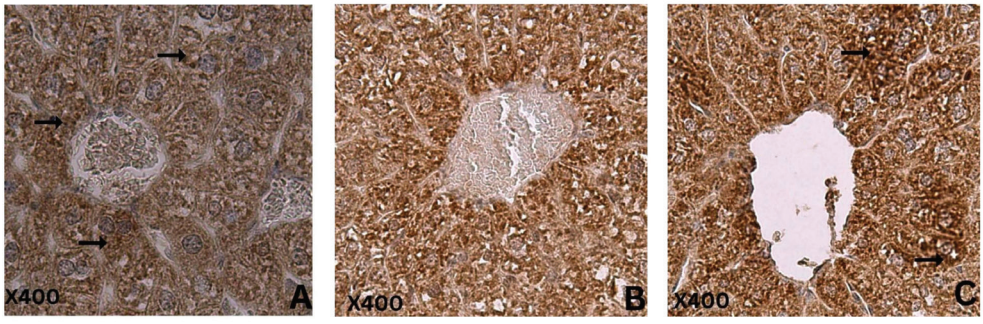

Fig. 3. Immunohistochemical expression of hepcidin in immature mouse liver: Control **(A)**, 75 mg/kg $CoCl_2$ **(B)**, 125 mg/kg $CoCl_2$ **(C)**. Hepcidin immunoreactivity in the hepatocytes (arrows). ×400

Expression of DMT-1 was observed in hepatocytes located near the central vein of the hepatic lobule, as well as in the endothelial cells of blood vessels. Notably, weaker protein expression of the protein was seen in liver sections from mice treated with a low dose of $CoCl_2$ **(Fig. 4)**, whereas treatment with high dose did not produce any changes in intensity of immunostaining. According to the literature, DMT-1 is expressed in liver hepatocytes and participates in the regulation of iron metabolism by importing iron bound to transferrin or non-transferrin-bound iron [19]. To date, the role of DMT-1 in hepatocytes *in vivo* concerning iron metabolism remains unclear and our data provide new knowledge to DMT-1 regulation in the liver. It is known that weak expression of DMT1 is associated with increased oxidative phosphorylation and glycolysis, which serves as an early signal for tumor recurrence in patients who have undergone surgery for hepatocellular carcinoma [8]. In addition to iron, DMT-1 also binds and transports other divalent metal ions, such as cobalt, which could explain altered expression in $CoCl_2$-treated animals.

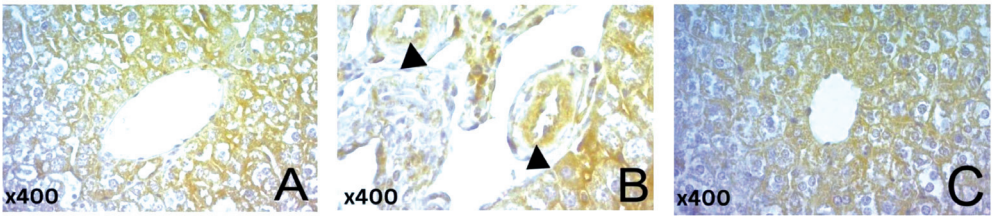

Fig. 4. Immunohistochemical expression of DMT1 in immature mouse liver: Control **(A)**, Blood vessel of the control **(B)**, 75 mg/kg $CoCl_2$ **(C)**. DMT1 immunoreactivity in the endothelial cells (arrowheads). ×400

Conclusion

Although cobalt is an essential trace element, it can cause serious damage when present in high concentration and with long-term exposure. In our study, prenatal and early postnatal exposure to $CoCl_2$ exerts a toxic effect on the liver, reducing the liver index and altering the activity of proteins involved in iron metabolism regulation. Chronic intake leads to significant iron accumulation in the liver, resulting from altered pattern of TfR1 protein expression and slightly suppressed expression of hepcidin. Our novel data on altered expression of iron regulatory proteins (TfR1, hepcidin and DMT-1) under chronic $CoCl_2$ exposure suggested that cobalt regulates their expression via diverse mechanisms. Enhanced erythropoiesis significantly reduces hepatic hepcidin production in both mice and humans, promoting the increased transfer of dietary iron and stored iron into the bloodstream for the synthesis of heme and hemoglobin by developing erythrocytes in the bone marrow [16]. Our study indicates that erythropoiesis plays a key role in regulating iron metabolism by inhibiting hepcidin production.

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References

1. Cattley, R., J. Cullen. Liver and Gall Bladder. - *In: Haschek and Rousseaux's Handbook of Toxicologic Pathology,* **3**, 2013, 1509–1566.

2. Drakesmith, H., E. Nemeth, T. Ganz. Ironing out Ferroportin. - *Cell Metab.,* **22**, 2015, 777-787.

3. Fisher, A. L., C.Y. Wang, Y. Xu, K. Joachim, X. Xiao, S. Philips, G. A. Moschette, V. M. Alfaro- Magallanes, J. L. Babitt. Functional role of endothelial transferrin receptor 1 in iron sensing and homeostasis. – *Am. J. Hematol.*, **97**, 2022, 1548-1559.

4. Genchi, G., G. Lauria, A. Catalano, A. Carocci, M. S. Sinicropi. Prevalence of Cobalt in the Environment and Its Role in Biological Processes. – *Biology (Basel)*, **12**, 2023, 1335.

5. Gleason, J. E., D. J. Corrigan, J. E. Cox, A. R. Reddi, L. A. McGinnis, V. C. Culotta. Analysis of hypoxia and hypoxia-like states through metabolite profiling. – *PLoS One*, **6,** 2011, 24741.

6. Gluhcheva, Y. G., V. N. Atanasov, J. M. Ivanova, E. H. Pavlova. Chronic exposure to cobalt compounds — an *in vivo* study. – C*ent. Eur. J. Biol.,* **9**, 2014, 973–981.

7. Graham, R. M., A. C. Chua, C. E. Herbison, J. K. Olynyk, D. Trinder. Liver iron transport. – *World J. Gastroenterol.,* **13,** 2007, 4725–4736.
8. Hoki, T., E. Katsuta, L. Yan, K. Takabe, F. Ito. Low DMT1 Expression Associates With Increased Oxidative Phosphorylation and Early Recurrence in Hepatocellular Carcinoma. – *J. Surg. Res.*, **234,** 2019, 343-352.
9. Ilovska, A. The effect of chronic treatment with cobalt chloride on iron metabolism in the liver of immature mice. *BSc thesis*, Sofia University "St. Kliment Ohridski", 2024, p.54.
10. Kincaid, R. L., M. T. Socha. Effect of Cobalt Supplementation During Late Gestation and Early Lactation on Milk and Serum Measures1. – *J. Dairy Sci.,* **90**, 2007, 1880–1886.
11. Muñoz-Sánchez, J., M. E. Chánez-Cárdenas. The use of cobalt chloride as a chemical hypoxia model. - *J. Appl. Toxicol.*, **39** (4), 2019, 556–570.
12. Nemeth, E., T. Ganz. Hepcidin and Iron in Health and Disease. – *Annu. Rev. Med.,* **74**, 2023, 261-277.
13. Rishi, G., V. N. Subramaniam. The liver in regulation of iron homeostasis. – *Am. J. Physiol. Gastrointest. Liver Physiol.,* **313**, 2017, 157-165.
14. Simonsen, L. O., A. M. Brown, H. Harbak, B. I. Kristensen, P. Bennekou. Cobalt uptake and binding in human red blood cells. - *Blood Cells Mol. Dis*., **46,** 2011, 266-276.
15. Smith, T. Human serum transferrin cobalt complex: Stability and cellular uptake of cobalt. – *Bioorg. Med. Chem.,* **13,** 2005, 4576–4579.
16. Srole, D. N., T. Ganz. Erythroferrone structure, function, and physiology: Iron homeostasis and beyond. – *J. Cell Physiol.,* **236,** 2021, 4888–4901.
17. Theurl, M., I. Theurl, K. Hochegger, P. Obrist, N. Subramaniam, N. Rooijen, K. Schuemann, G. Weiss. Kupffer cells modulate iron homeostasis in mice via regulation of hepcidin expression. – *J. Mol. Med. (Berl)*, **86,** 2008, 825-835.
18. Vogt, A. S., T. Arsiwala, M. Mohsen, M. Vogel, V. Manolova, M. F. Bachmann. On Iron Metabolism and Its Regulation. – *Int. J. Mol. Sci*., **22,** 2021, 4591.
19. Wang, C. Y., M. Knutson. Hepatocyte divalent metal-ion transporter-1 is dispensable for hepatic iron accumulation and non-transferrin-bound iron uptake in mice. – *Hepatology,* **58,** 2013, 788-798.
20. Zou, H., J. Sun, B. Wu, Y. Yuan, J. Gu, J. Bian, Z. Liu, Z. Liu. Effects of Cadmium and/or Lead on Autophagy and Liver Injury in Rats. – *Biol. Trace Elem. Res.,* **198,** 2020, 206-215.