THE EXPRESSION OF IMMUNOHISTOCHEMICAL MARKERS IN THE FETAL LIVER AFTER MATERNAL EXPOSURE OF LEAD ACETATE AND UNDER THE CORRECTION

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Abstract. *The purpose of the study was to determine the expression of immunohistochemical markers in the fetal lever after lead acetate treatment and under the correction. We used the intact Vistar rat fetuses at 14th, 16th and 18th days of prenatal development, after maternal lead acetate treatment (50 mg/kg) alone and together with lycopene (500 mg/kg) for 3 weeks before pregnancy and during pregnancy. The expression of AE1/AE3, VEGF, MMP-1 and MMP-9 was significantly suppressed by toxicant and slightly corrected. eNOS expression revealed that the small vessels were the most vulnerable with a low potential for the correction. Lead acetate did not change the αSMA expression itself, but the accumulation of αSMA-positive Ito cells was depended on the degree of dystrophic and fibrotic changes in the liver. The expression of WT1 did not reveal the susceptibility to lead acetate, whereas caspase-3 expression increased.*

Keywords: *fetal liver, lead acetate, immunohistochemical markers, lycopene, rat.*

Lead is one of the oldest and most widespread industrial toxicant, which is present in the air, water and food. The harmful influence of lead causes the blockage of many enzymes [1]. It is also known as an agent, which can easily get through the placental barrier and impair the prenatal development of the organs and systems [1, 2]. The researches of prenatal development after maternal exposure of lead acetate started about forty years ago [2]. It has been proved that lead acetate treatment before and during pregnancy leads to varied aberrations and the liver has turned to be one of the most vulnerable organ [3, 4]. The previous studies of immunohistochemical markers expression have been mostly referred to the molecular-biological features of the liver in normal ontogenesis [5] and the effects of lead on the mature liver [4, 6]. The addition of vitamin E, coenzymes, sorbents, seaweeds and some plants to food [3, 4, 6, 7, 8] was proposed as the ways for the prevention and treatment of chronic lead intoxication. In our previous researches, we had showed the gradient of structural changes in the rat fetal liver as well as the inhibition of synthetic potential of the fetal liver under lead acetate treatment before and during pregnancy [3, 9, 10]. There has still been a shortage of data about the molecular changes in prenatal hepatic ontogenesis after lead acetate treatment and under the correction.

The purpose of the current study was to determine the expression of spectrum of immunohistochemical markers in the fetal lever after lead acetate treatment and under the correction.

Materials and methods. The materials were the livers of the intact Vistar rat fetuses at 14th, 16th and 18th days of prenatal development, after lead acetate treatment and after lead acetate treatment with lycopene (Hubei Pharmaceutical). Female rats were treated by 50 mg/kg of lead acetate per os daily for 3 weeks before pregnancy and during pregnancy, the group under correction was treated by the same dosage of lead acetate and lycopene (500 mg/kg). The fetuses were taken, fixed in 10 % formalin and embedded into paraplast. The sections (5 μm thick) were mounted on the SuperFrost Plus glass slides.

In order to renew the antigenic properties of the tissue, the thermal induction of the epitope restitution was performed by heating the slides in citrate buffer after their deparaffinization. The incubation with primary antibodies was carried out in humid chambers at a temperature of 23-25° C for 30 min. The antibody spectrum included the markers (polyclon, LabVision): αSMA (αsmooth muscle actin), eNOS (endothelial or constitutive nitric oxide synthase), epithelial marker AE1/AE3 (AE1 reveals acidic cytokeratins, AE3 recognizes all known basic cytokeratins), VEGF (vascular endothelial growth factor), nuclear marker WT1 (Wilm's tumour gene), apoptotic marker caspase-3, MMP-1, MMP-9 (matrix metalloproteinase-1 and matrix metalloproteinase-9). The antibody titer was individually selected for each marker. The next stage of the immunohistochemical study was performed using UltraVision Quanto (LabVision) imaging system. The identification was carried out by applying DAB chromogen under the microscope control from 20 sec to 3 min until the dark brown coloring of specific structures manifested. The sections were additionally stained by Mayer's hematoxylin.

The experiments were carried out in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986) and the Law of Ukraine "About the Protection of Animals from Cruel Treatment" (2006).

Results. In the control group, AE1/AE3 as an epithelial marker demonstrated high intensive immunostaining in hepatocytes and was able to identify clearly the areas of the liver parenchyma for 14-18th prenatal days. We have not observed the heterogeneity in AE1/AE3 expression in hepatocytes. The eNOS-positive cells in the fetal liver were the endothelial cells of interstitial blood vessels, central veins and other vessels, except for the sinusoidal endothelium. The cells of the vessels wall, as well as the stellate cells (Ito) were αSMA-positive. There has been intense hematopoiesis around the middle and large blood vessels, and hematopoietic islets were based on αSMA-positive cells. Ito cells were sparse and mostly isolated.

The hepatocytes were strongly VEGF-positive; the granules of stain were distributed by relatively uniform pattern in the cytoplasm of the hepatic epithelium. The nuclei of some hepatocytes were weakly caspase-3-positive, whereas the reaction in the nuclei of stromal cells was more intensive. MMP-1 and MMP-9 mostly expressed in the stromal cells, and some hepatocytes were MMP-9-positive from 14th until 18th days as well. The nuclei of hepatocytes were moderately WT1-positive the whole period.

In the group after lead acetate treatment, the changes in the liver parenchyma included the dystrophy; some of hepatocytes underwent almost complete vacuolization. The pathological processes were enhancing during 14-18th prenatal days. We also observed the suppression of hematopoietic function of the liver right up to total disappearance of hematopoietic islets. The decreasing of AE1/AE3 expression correlated to the general degenerative phenomena in the liver parenchyma; the intact hepatocytes showed the less immunostaining with the excessive heterogeneity in AE1/AE3 expression.

The significant effects of the toxicant on the vascular endothelium revealed itself in eNOS expression. The inhibition of the eNOS synthesis led to a barely noticeable accumulation of stain in the vascular endothelium at 14th day. Further observations showed a stable suppression of the synthesis of this enzyme. The number of αSMA-positive interstitial cells, which we believed to be stellate cells [10], was increasing since 14th prenatal day. The intensive proliferation of these cells, which tended to congregate, was observed in the peripheral regions of the liver undergoing the most severe pathological changes. αSMA-positive cells were mostly located along the sinusoids. At 18th prenatal day, the most number of these cells was observed in the peripheral parts of the organ, where hepatic parenchyma was still relatively intact. The areas with rough fibrotic changes excluded the presence of Ito cells. αSMA-positive cells in these areas were observed only in the blood vessels that began to form in the fibrous tissue.

The cytoplasm of hepatocytes was VEGF-positive, but the expression of this marker became non-uniform. The immunostaining pattern varied from weak to intensive in liver parenchyma, whereas the stromal cells showed the strong VEGF expression. The hepatocytes with caspase-3-positive nuclei were more numerous at 14th prenatal day and its number had been increasing until 18th day. The nuclei of stromal cells were caspase-3-positive the whole period. There were no changes in WT1 expression. The inhibition of MMP-1 and MMP-9 expression in the liver was an evidence for the deep impairment of the stromal component.

In the group after lead acetate and lycopene treatment, the expression of the AE1/AE3 was suppressed a bit less than in the group without correction, but never reached the intensity observed in the control group. The AE1/AE3 immunostaining also lost its uniform pattern in the cytoplasm of hepatocytes. The eNOS expression was reduced, the distribution of stain showed that the smallest vessels were the most vulnerable in this group. The areas with the significant dystrophic and necrotic changes lost the usual vascular pattern. Nevertheless, there were some vessels of medium caliber with the sufficient level of synthesis of enzymes in areas with varied degree of impairment of hepatic parenchyma.

The distribution of αSMA-positive cells resembled to some extend the intact liver, but in the areas undergoing significant dystrophic changes we noticed an increased number of these cells. We observed the accumulation of αSMA-positive cells along the sinusoids by 18th prenatal day, as well as near the structures resembling sinusoids, which, however, lost their features, including the endothelium.

The VEGF expression in the cytoplasm of hepatocytes was similar to the group after lead acetate treatment. The stromal cells demonstrated high VEGF expression, especially in the areas with fibrotic changes. WT1 expression showed no changes. Caspase-3-positive hepatocytes were more numerous in the peripheral parts of the organ, whereas caspase-3-positive stromal cells were ubiquitous. The MMP-1 and MMP-9 expression was similar with the group after lead acetate treatment.

Discussion. After lead acetate treatment, the changes in the liver parenchyma were more expressive that we had noticed before, when we used the half less dosage of lead acetate [3]. The

morphological manifestations of the pathological processes in the liver parenchyma were aggravating during prenatal period. The weak expression of epithelial marker as AE1/AE3 is an evidence for the deep inhibition of protein synthesis in the fetal liver with a slight tendency to be corrected by lycopene. The decrease of the synthesis of the constitutive form of nitric oxide synthase, especially in small caliber vessels, corresponded the overall toxic effect of lead, which was also observed in the mature liver [4, 6]. The protector used partially masked the influence of lead on eNOS expression.

We noticed the certain stimulation of the αSMA-positive cells proliferation by the toxicant. The concentration of these cells, which were proved to be Ito cells, was maximal in the areas with the dystrophic changes. In our studies, the accumulation of αSMA-positive cells at 14-18th days of prenatal development was accompanied with the further fibrotic changes, especially in the peripheral parts of the organ, but after 18th prenatal day, the areas, where the connective tissue had replaced the liver parenchyma, contained no longer Ito cells [10]. Therefore, we believe that Ito cells, even in the prenatal period, are associated with fibrotic processes in the liver; they undergo apoptotic changes after the formation of fibrous tissue.

The expression of growth factors, in particular VEGF, demonstrated more expressed susceptibility to lead acetate in the liver parenchyma compared to the stroma. Caspase-3 is essential for the nuclear changes associated with apoptosis, and the increasing of caspase-3 expression was certainly associated with the degree of morphological changes.

The inhibition of metalloproteinases (MMP-1 and MMP-9), which are important for the extracellular matrix, was revealed in both the liver parenchyma and stroma. The activation of apoptotic processes after lead acetate treatment was significant and seemed to be faintly corrected with lycopene. The WT1 expression was not depended on toxicant, so we could regard the expression of this marker to be the most stable during fetal liver development.

Conclusions. The expression of immunohistochemical markers in the fetal liver showed different susceptibility to lead acetate and potential for the lycopene correction. The expression of AE1/AE3, VEGF, MMP-1 and MMP-9 was strongly suppressed by toxicant and slightly corrected. eNOS revealed the more expressive vulnerability of small vessels with low potential for the correction. Lead acetate did not change the αSMA expression itself, but the accumulation of αSMA-positive Ito cells was depended on the degree of morphological changes in the liver, both dystrophic and fibrotic. The WT1 expression did not reveal the susceptibility to lead acetate, whereas caspase-3 expression increased.

REFERENCES

1. Uzbekov M. G. Effect of prenatal lead exposure on superoxide dismutase activity in the brain and liver of rat fetuses // Uzbekov M. G., Bubnova N. I., Kulikova G. V. // Bull. Exp. Biol. Med. – 2007. – Vol. 144, № 6. – P. 783-785.

2. Fetal rat development as influenced by maternal lead exposure / Miller C. D., Buck W. B., Hembrough F. B., Cunningham W. L. // Vet. Hum. Toxicol. – 1982. – Vol. 24, № 3. – P. 163-166.

3. Dovhal H. V. Morfolohichni zminy v rozvytku pechinky shchuriv pry vplyvi atsetatu svyntsiu ta za umov korektsii v prenatalnomu periodi / Dovhal H. V. // Ukrainskyi morfolohichnyi almanakh. – 2014. – T.12, № 1. – S. 42-44.

4. Kupsha A. I. Morfo-funktsionalnyye proyavleniya kompensatorno-prisposobitelnykh reaktsiy stromalnogo komponenta pecheni belykh myshey pri dlitelnom postuplenii v organizm malykh doz svintsa i ikh korrektsiya / A. I. Kupsha. O. M. Grabovoy // Nauchnyy vestnik Natsionalnogo meditsinskogo universiteta imeni O. O. Bogomoltsa. — 2008. — № 4. — S. 57-63.

5. Cell growth and differentiation of different hepatic cells isolated from fetal rat liver in vitro / Fiegel H. C., Bruns H., Höper C. [et al.] // Tissue Eng. – 2006. – Vol. 12, № 1. – P. 123-130.

6. Kupsha E. I. Morfo-funktsionalnoye sostoyaniye pecheni pri svintsovoy intoksikatsii i korrigirovaniya protsessa tokoferolom i erbisolom / E. I. Kupsha. N. K. Kashirina // Tavricheskiy mediko-biologicheskiy vestnik. — 2004. — T. 7. № 4. — S. 64-69.

7. Dandelion-enriched diet of mothers alleviates lead-induced damages in liver of newborn rats / Gargouri M., Magné C., Ben Amara I., Ben Saad H., El Feki A. // Cell Mol. Biol. – 2017. – Vol. 63, № 2. – P. 67-75.

8. Spirulina exhibits hepatoprotective effects against lead induced oxidative injury in newborn rats / Gargouri M., Ben Saad H., Ben Amara I., Magné C., El Feki A. // Cell. Mol. Biol. – 2016. – Vol. 62, № 10. – P. 85-93.

9. Dovhal H. V. Vplyv atsetatu svyntsiu na vyzhyvanist zarodkiv shchuriv ta mozhlyvist korektsii / Dovhal H. V. // Visnyk problem biolohii i medytsyny. – 2014. – Vyp. 1 (105). – S. 93-96.

10. Romanenko O. A. Imunohistokhimichne doslidzhennia pechinky shchuriv v piznomu prenatalnomu periodi pid vplyvom atsetatu svyntsiu ta za umov korektsii / Romanenko O. A., Dovhal H. V., Dovhal M. A. // Visnyk problem biolohii i medytsyny. – 2012. – Vyp.3. – S. 158-161.