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CONTENT OF METALLOTHIONEINS IN THE ORGANS OF SHEEP UNDER CHRONIC INTAKE OF LEAD WITH RATION

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Abstract

When studying the effects of lead on mammals, one of the informative indicators is the level of metallothioneins (MTs) in organs. MTs are low molecular weight proteins (6-7 kDa) containing up to 30 % cysteine which bind heavy metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+}) and act as a trap for free radicals. It is assumed that the MTs content in tissues of mammals depends not only on the amount of lead, the way of intake (with water and/or diet), the type of the tissue and its physiological functions, but on the number of cells in the body and their functional activity. At the same time, the violation in functional and synthetic activity of cells may be related to changes in the weight indices of the bodies. In this regard, the study of MT levels in farm animals as influenced by lead is of interest. In the present work we studied experimentally the MT content, the cellularity and the weight index of the organs in sheep of the Romanov breed under lead intoxication. The experiment was conducted on 27 animals of 33.0±1.1 kg in weight at the age of 1-1.5 years. The sheep were divided into four groups. The group I (4 intact animals) served as a control. For 90 days the sheep in groups II (5 animals), III (9 animals) and IV (9 animals) had a daily diet with lead nitrate at 5 mg/kg, 25 mg/kg and 150 mg/kg of feed, respectively, which corresponded to 1, 5 and 30 MPL (maximum permissible levels) of the metal in the feed. The diet consisted of 0.3 kg of concentrate and 2 kg mixed grass hay. Lead nitrate was administrated with feed once a day. Daily intake of metal (on average per animal) was 10 mg in group II, 50 mg in group III, and 300 mg in group IV, and the dose was 0.3, 1.5 and 9 mg/kg of animal weight, respectively. The study of biological indices was performed in the organs with different physiological functions (liver, kidneys, spleen), which vary in the intensity of metal accumulation and proliferation. The liver, kidneys and spleen were collected after slaughter before the beginning of the experiment (before priming) in one animal, then on days 30 and 60 of intoxication in one animal from group II, 3 animals from group III and 3 animals from group IV, and on day 90 in 3 animals from each group. It was found that constitutive MT levels differed, being higher in sheep liver and kidneys when compared to spleen, while the cell number was less. As a result of chronic intake of lead from diet, the content of MTs in the liver, the number of cells and the body weight index increased. The most pronounced changes were observed in the animals of group IV. In the kidneys there were non-linear changes in the MT level. Decrease in the MT content on day 30 of the intoxication as cell number in kidneys increased was indicative of inhibition of the cell synthetic activity, and an increase in body weight index at days 60 and 90 while the cell number reduced indicated the development of negative processes. In the spleen, the MT synthesis was inhibited, and non-linear changes in the cell number were observed. Comparison of the obtained results allows us to assume the development of negative processes in the liver, kidneys and spleen that is associated, on the one hand, with intensity of lead accumulation, and on the other hand, with the sensitivity of cells in the organs to toxic effects of this metal. It is assumed that low constitutive MT level is one of the factors of high sensitivity to lead in splenocytes.

Keywords: lead, metallothionein, sheep, liver, kidney, spleen.

Lead as an environmental pollutant and a classic toxic element attracts the attention not only of hygienists and toxicologists but also of ecologists [1-4]. Lead enters mammal bodies with food and water and is accumulated in their organs and tissues. When studying the effects of lead on mammals, one of the informative indicators is the level of metallothioneins (MTs) in organs [5-8].

MTs are low molecular weight proteins (6-7 kDa) containing up to 30 %

cysteine. In mammal organisms, MTs bind heavy metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+}), maintain homeostasis of copper and zinc, and act as a trap for free radicals [9-12]. According to the degrees of MT synthesis induction in mice liver the metal ions are arranged as $Cd^{2+} > Pb^{2+} > Zn^{2+} > Hg^{2+} > Cu^{2+}$ [13].

MT synthesis activation in the tissues of laboratory animal organs has been demonstrated to be dependent on the way of lead intake. Thus, the expression of MTs binding lead and zinc in the tissues of mice organs was detected at intravenous and intraperitoneal administration of lead acetate, but not at subcutaneous administration [14]. The increased level of MTs in rat kidney tissue was observed in chronic lead intake with drinking water at the concentrations of 200-300 mg/l [15].

In farm animals, in sheep particularly, chronic lead nitrate intake from diet at the doses of 5, 25, and 150 mg/kg feed resulted in the increased MT levels in peripheral blood lymphocytes [16]. At the same time, there are no data on MT synthesis in the organs of farm animals with lead intake from diet. Moreover, the constitutional levels of the synthesis of these compounds in sheep organs with different physiological functions which vary in the intensity of lead accumulation and proliferation, have not been determined. Thus, liver and kidneys have low proliferative activity and accumulate more lead compared to spleen [17-21].

It is assumed that the MT content in mammal tissues depends not only on the amount of lead, the way of its intake, the tissue type and its physiological functions, but on the number of cells in the organ and their functional activity (synthesis intensity and MT gene expression). In chronic lead exposure, the changes in the functional and synthetic activity of mammal cells may be followed by a decrease in their viability and (or) proliferation, which results in the changes of the organ weight index (the organ-to-body weight ratio) changes.

In this study, we first estimated the level of constitutive MT synthesis in the organs with different functions, and the induced MT expression under chronic lead intoxication coming from the diet, compared with the state and proliferative activity in these organs, in Romanov breed sheep. Comparative analysis revealed the differences in MT content in organs. In general, the growth of toxic load was followed by negative processes: MT synthesis reduction with the increase in the number of cells and in the relative organ weight. A low level of constitutive MT synthesis may be one of the factors of high splenocyte sensitivity to lead.

The purpose of this study was to estimate the metallothionein content, the cellularity and the weight index of the liver, kidneys, and spleen in sheep under chronic lead intake from diet.

Technique. The studies were performed in 27 Romanov breed sheep (body weight of 33.0±1.1 kg, age of 1-1.5 years). Feeding and animal care were performed in accordance with the «Regulations of work with the use of experimental animals» (Order of the Health Ministry of Russia of 08/23/2010 № 708 n). Sheep were kept in the All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals vivarium (Borovsk, Kaluga Province), fed twice a day and had unlimited access to water. The diet consisted of 0.3 kg of feed and 2 kg of mixed grass hay. Compound feed contained 44.0 % of barley, 41.4 % of wheat, 11.7 % of sunflower meal, 1.0 % of sodium chloride, 1.0 % of defluorinated phosphate, 1.0 % of premix; hay composition: 87.9 % of crude material, 8.89 % of protein, 2.26 % of fat, 32.6 % of dietary fiber, and 4.26 % of ash.

The sheep were divided into four groups. Group I (4 intact animals) was a control group; the group II (5 animals), III (9 animals), and IV (9 animals) sheep were fed a daily diet with lead nitrate (5 mg/kg, 25 mg/kg, and 150 mg/kg of feed, respectively) for 90 days. Lead content in the group II diet corresponded

to 1 MPL (maximum permissible level) of metal in animal feed; in group III it was 5 MPLs, and in group IV it was 30 MPLs. Lead nitrate was given with compound feed once a day based on the amount of feed (average of 2 kg) entering the gastrointestinal tract. For this purpose, 100 g of this compound feed were mixed with 50 ml of lead nitrate solution of the desired concentration. The average daily intake of metal per animal was 10 mg in group II, 50 mg in group III, and 300 mg in group IV, and the doses were 0.3, 1.5 and 9 mg/kg of animal body weight, respectively. Organs (liver, kidney, spleen) were collected (after the slaughter) prior to the beginning of the experiment (before priming) in 1 animal of group I, at the days 30 and 60 of intoxication in 1 animal of group II and 3 animals of groups III and IV, and at the day 90 in 3 animals of each group.

MT content in the organ tissues was estimated by a radiochemical method [22, 23] based on the substitution of ^{109}Cd metal ions chelated to MTs. Tissue samples were homogenized in Tris-HCl buffer (0.1 M, pH 8.2) and diluted to the volume of 2 ml. To denaturate macromolecular proteins, the supernatant was heated in a boiling water bath for 3 min, cooled on ice and centrifuged for 7 min at 16,000 g and 4 °C. An aliquot of 0.2 ml of the sample was added to 0.2 ml of the indicator reagent, mixed and incubated for 10 minutes at 20 °C. To prepare the indicator reagent, 40 μl of $^{109}CdCl_2$ and 20 μl of $CdCl_2$ (concentration of 100 μg/ml) were added to 1 ml of Tris-HCl buffer. 0.1 ml of 2 % hemoglobin reference solution (Agat Co., Russia) was added in the tubes with samples, stirred and heated in a boiling water bath for 3 minutes followed by cooling on ice. The similar procedure was performed twice. Then the samples were centrifuged for 15 minutes at 3900 g and 4 °C and the supernatant was collected (0.2 ml).

A blank sample (0.2 ml of buffer added instead of the test sample) and total activity (the test sample and hemoglobin were changed by buffer, the reference) were analyzed for each series of measurements .

To calculate the MT amount in the samples, the following formula was used [22]:

$$M = [17.8 \cdot V \cdot (A - K) \cdot C^{-1}] \cdot m^{-1},$$

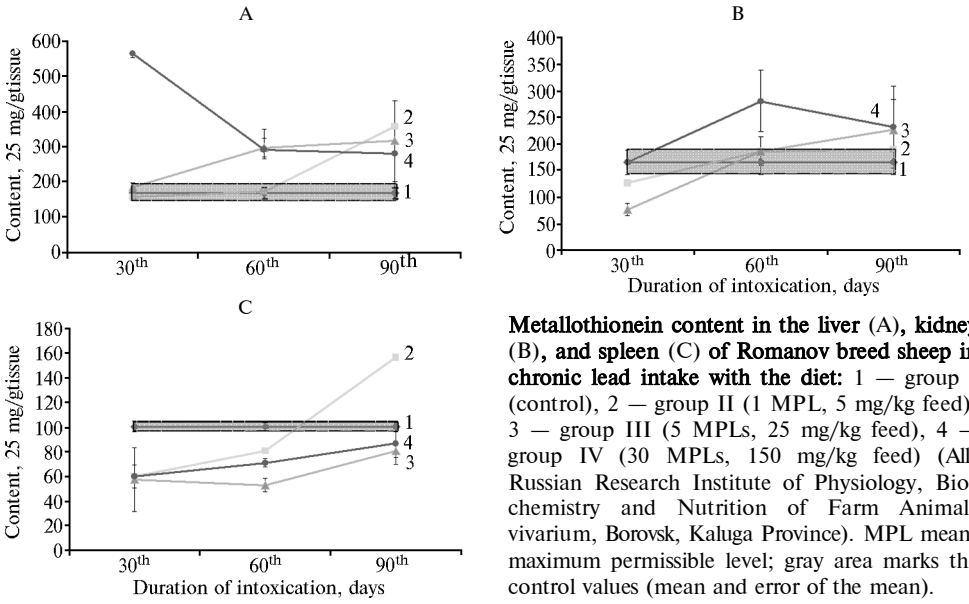
where M was MT amount, μg/g tissue; V was a multiplicity of sample dilution; 17.8 was cadmium amount in the sample, nmol/ml which corresponds to the MT amount in the sample in μg (the value used for conversion); m was sample weight, g; A, K, C were the decay number in the samples, blank sample and reference, respectively. Radioactivity of samples was measured using a low energy γ-spectrometer with semiconductor detector SO-05P1 (Aspect, Russia).

Cellularity and the organ weight index were estimated by standard methods [24]. Preweighed biomaterial from the middle part of an organ was homogenized with a teflon pestle in a test tube with a small volume of medium (140 mM NaCl, 5 mM KCl, 5 mM HEPES buffer, pH 7.4). The medium volume was 2 ml for the kidneys and liver, and 4 ml for the spleen. Haemolysis of erythrocytes in cell suspensions was performed using Turk's solution. The content of the tubes was mixed thoroughly and the supernatant was collected in 2 minutes for cell counting in the Goryaev's camera. Organ cellularity was estimated by the following formula: $K = Y/m$, where m was sample weight, mg; Y was cell number. To calculate the organ weight index (coefficient), the following formula was used: $I = m_1/M_1$, where m_1 was organ weight, kg; M_1 was sheep body weight, kg.

The statistical processing of the results was carried out using the variation statistics method with Student's *t*-test. The differences in the values were considered significant at $p < 0.05$ [25].

Results. MT content in intact sheep (control) was 168±16 μg/g tissue (Fig.,

A). In the group II animals (1 MPL), the index value was similar to the control within the first 60 days of intoxication, and at the day 90 it was 2.1 times higher versus control ($p < 0.05$). With the increase of metal intake with the diet, the MT amount in the liver of group III (5 MPLs) and group IV (30 MPLs) sheep was significantly increased in all study periods.



Metallothionein content in the liver (A), **kidney** (B), **and spleen** (C) **of Romanov breed sheep in chronic lead intake with the diet:** 1 — group I (control), 2 — group II (1 MPL, 5 mg/kg feed), 3 — group III (5 MPLs, 25 mg/kg feed), 4 — group IV (30 MPLs, 150 mg/kg feed) (All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals vivarium, Borovsk, Kaluga Province). MPL means maximum permissible level; gray area marks the control values (mean and error of the mean).

Thus, in group III animals, this parameter in days 30, 60, and 90 was 11 %, 77 % ($p < 0.05$), and 88 % ($p < 0.05$) higher versus control. At the same time, the maximum amount of MTs in the group IV sheep liver was recorded at the day 30 of intoxication (335 % of control). At days 60 and 90, a reduction in MT amount in this organ was observed, although the values were significantly higher, by 73 and 66 % ($p < 0.05$), respectively, than in control.

Cellularity and organ weight index in Romanov breed sheep depending on the doses and the timing of chronic lead intake from diet (*M*±*m*, All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals vivarium, Borovsk, Kaluga region)

Group	Pb^{2+}, mg/kg feed	Cellularity, $\times 10^3$ cells/mg tissue			Organ weight index, $\times 10^{-4}$		
		30 days	60 days	90 days	30 days	60 days	90 days
		Liver					
I	0	Average value 14.3±1.0			Average value 11.2±0.4		
II	5	23.1	28.6	16.1±1.4	16.1	14.9	11.8±0.3
III	25	21.7±0.7*	29.4±5.1*	39.4±21.5	14.3±0.7*	14.2±1.0*	11.1±0.4
IV	150	35.7±1.9*	37.7±9.2*	37.7±5.9*	15.6±0.9*	14.8±1.2*	12.0±0.3
		Kidneys					
I	0	Average value 14.0±0.9			Average value 19.5±1.2		
II	5	12.3	16.3	18.8±1.2*	22.6	19.7	20.9±0.5
III	25	18.7±1.1	14.7±1.1	11.8±7.9	24.0±1.2*	21.5±0.5	22.2±0.5
IV	150	16.2±7.9	11.9±1.2	8.4±0.2	23.6±0.9*	24.8±0.5*	37.2±7.4*
		Spleen					
I	0	Average value 35.2±0.5			Average value 12.0±2.0		
II	5	48.2	27.8	42.7±1.6*	12.0	10.3	11.3±1.4
III	25	64.6±1.0*	36.1±2.3	31.1±4.3	11.5±0.9	12.1±1.2	12.2±1.2
IV	150	62.6±7.0*	60.9±4.9*	33.1±3.0	13.8±1.7	10.8±1.3	12.4±1.1

Note. For description of groups, please see section Technique.
* Differences with the control were significant at $p < 0.05$.

Indeed, the number of cells in the liver increased by 62 and 100 % in group II animals at days 30 and 60, respectively (Table). The most pronounced changes in the parameter were observed with the increase in the amount of

metal in the diet. The number of cells increased by 52 % ($p < 0.05$), 106 % ($p < 0.05$), 176 % and 150 % ($p < 0.05$), 164 % ($p < 0.05$), 164 % ($p < 0.05$) in the group III and IV sheep liver tissue at days 30, 60, and 90, respectively. At the same time, the organ weight index increased in the experimental animals in almost all periods of the study (Table). A significant increase of these values was observed at days 30 and 60 in the III and IV group sheep. The most pronounced changes were observed in the animals of group IV.

Our results demonstrated a compensatory reaction of the organism, however, a decrease in MT content in group IV sheep liver tissue at days 60 and 90 with an increase in the number of cells in the organ suggests the inhibition of hepatocyte synthetic activity and the development of negative processes. Our results on lead accumulation in the liver during these periods are in line with this finding (data not shown).

MT amount in intact sheep kidneys (Fig., B) was 166±23 μg/g tissue. In the II group animals, this parameter values were lower than in control. Later, the MT amount increased. The similar tendency was recorded with the increase of lead content in the diet. Thus, a 54 % decrease versus control ($p < 0.05$) was observed in group III sheep at day 30. This parameter increased at days 60 and 90. At the same time, MT level was not significantly different from control values in group IV animals at the day 30 of observation, and at days 60 and 90 it was by 69 % ($p < 0.05$) and 39 % higher, respectively, compared to control. Low values of the parameter at the day 30 of intoxication could be explained by the inhibition of MT synthesis in the cells, by the MT isoform specificity and by the organ cellularity. Thus, the number of kidney cells in group II animals increased by 34 % ($p < 0.05$) at day 90 of intoxication (see Table). This organ cellularity was greater versus control in group III and IV sheep at day 30 (34 % and 16 %). Later, the cell number tended to decrease. At day 90, it was 16 and 40 % lower than in control, respectively. Probably, the intergroup differences observed were dependent on the content of the metal in the organ. The low amount of lead in the kidneys is assumed to activate proliferative processes, and the high amount, in contrast, is suggested to cause cell death.

Estimation of kidney weight index demonstrated an increase of this parameter (see Table). Significant differences with control were recorded at day 30 in group III animals, and during all periods of the study in group IV sheep.

It should be emphasized that the changes in the kidney MT content were nonlinear. A decline of this parameter at the day 30 of intoxication with the increase in the cell number makes it possible to discuss the suppression of their synthetic activity and the development of negative processes. This is supported by the data on the increase in the organ weight index followed by the reduction in the cell numbers at days 60 and 90.

MT content in the group I spleen tissue was 100.5±4.4 μg/g (Fig., C). This parameter value reduced by 40 % ($p < 0.05$) and 20 % in the group II animals at the days 30 and 60 of intoxication, respectively. At day 90, MT content was significantly higher versus control, by 56 % ($p < 0.05$). With increased lead amounts in the diet, low values of the parameter were recorded almost in all periods of the study. Thus, MT synthesis intensity was inhibited by 43 % ($p < 0.05$), 47 % ($p < 0.05$) and 20 % ($p < 0.05$) in group III, and by 40 % ($p < 0.05$), 30 % ($p < 0.05$) and 14 % in group IV, respectively.

The number of cells in the spleen in group II sheep was by 37 % higher at intoxication day 30 than in control (see Table). At day 60, there was a tendency to a decrease in the value of this parameter, although the number of cells in the organ increased significantly by day 90. In the group III and IV sheep, the values were greater versus control within the first 60 days, and then decreased.

Determination of spleen weight index identified no significant differences in any groups compared to control.

Nonlinear dynamics of the number of cells in the spleen in the absence of the changes in the weight index was indicative of the formation of compensatory reactions. However, inhibition of MT synthesis in splenocytes was rather indicative of the development of negative processes.

Comparative analysis revealed the differences in MT content in organs. MT amount in sheep liver and kidneys was higher than in the spleen. In chronic lead intake from the diet, an increase in the parameter value was observed in the liver, while in the spleen, on the contrary, it reduced. The changes in MT amount in kidneys were nonlinear. Probably, the basal MT levels in organ cells characterize their sensitivity to lead exposure. At the same time, the intensity of MT synthesis at the same lead concentration can vary [26, 27]. Furthermore, the increase of MT content in an organ may be associated with regeneration and/or proliferation.

Indeed, the intact sheep cellularity was $(35,2\pm0,5)\times10^4$ cells/mg tissue in the spleen, $(14,0\pm0,9)\times10^4$ cells/mg tissue in the kidneys, and $(14,3\pm1,0)\times10^3$ cells/mg tissue in the liver. Hence, the following sequence can be represented according to the number of cells in the organ: spleen > kidneys > liver. With lead intake, the changes in the organs were of multidirectional nature. The number of liver cells increased during the entire study period, spleen and kidney cellularity increased at day 30, after which a decrease was observed. The intensity of lead accumulation in liver and kidney tissues was greater than in the spleen, and the results therefore suggest the splenocyte sensitivity. A low level of constitutive MT synthesis may be one of the factors of high splenocyte sensitivity to lead. It should be stressed that in chronic lead intake, the organ weight index change was observed in the liver and kidneys only.

Thus, chronic lead intake from the diet at the doses of 5 (1 MPL), 25 (5 MPLs), and 150 mg/kg feed (30 MPLs) resulted in an increase in the metallothionein (MT) level in the liver, as well as in the increase in the number of cells and the organ weight index. The most significant disorders were observed in the animals of group IV (30 MPLs). The changes in the kidney MT content were nonlinear. Its decrease at day 30 with an increase in the number of kidney cells is indicative of inhibition of the cell synthetic activity, and the organ weight index growth followed by a reduction in cell numbers at days 60 and 90 suggests the development of negative processes. In the spleen, MT synthesis was inhibited, and nonlinear changes in the cell number were observed. Our results indicate the development of negative processes in the liver, kidneys and spleen, which is associated, on the one hand, with the intensity of lead accumulation and on the other hand, with the sensitivity of cells in the organs to toxic effects of this metal. MT content in liver and kidney tissues in intact sheep was greater than in the spleen, and the number of cells was lower. A low level of constitutive MT synthesis may be one of the factors of high splenocyte sensitivity to lead.

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