Kinetic models for the lead bioaccumulation and mitotic index in lead-poisoned and zeolite-treated laboratory mice

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Abstract

Clinoptilolite sorbent KLS-10-MA versus lead toxicity was applied for the first time. The dietary inclusion of the sorbent reduced Pb concentration in the exposed and supplemented laboratory mice by 84%, 89%, 91%, 77% and 88%, in carcass, liver, kidneys, bones, and feces, respectively. There were observed 3.8-fold higher chromosome aberrations frequency (CAF), 2.9-fold lower mitotic index (MI), 2.3-fold more pathological erythrocytes, and 1.3-fold lower body weight; and 1.9-fold higher CAF, 1.16-fold lower MI, 1.9-fold more pathological erythrocytes, and 1.03-fold lower body weight toward the Control group, in the Pb-poisoned; and in the Pb-poisoned and supplemented mice, respectively.

A mathematical model was proposed to outline the common trends of the Pb kinetics in the animals. The coefficient of absorption of Pb by gastrointestinal mucosa in the supplemented mice was found: $\eta = 3.53\%$ (versus $\eta = 15\%$ in non-supplemented ones).

For the first time a mathematical model was constructed for mitotic index change in conditions of chronic intoxication. The model clearly shows that the recovery processes in the animals run in parallel with the Pb bioaccumulation and that the susceptibility of the mouse's organism to Pb load decreases and the recovery rate of the genetic apparatus increases during the experiment.

Keywords: Lead bioaccumulation, Clinoptilolite sorbent, Laboratory mice, Chromosome aberrations, Mitotic index, Erythrocytes, Body weight, Mathematical model for Pb bioaccumulation, Mathematical model for mitotic index

1. Introduction

Zeolites, especially clinoptilolites, are widely used for removing toxic substances from different solutions and wastewater. Our idea was to apply clinoptilolite as antidote versus metal load in living organism. Here clinoptilolite sorbent, as a food additive, was tested in conditions of lead (Pb) intoxication in laboratory mice.

Lead – one of the most common anthropogenic pollutants, resulting from anthropogenic activities – is markedly deleterious factor for living organisms. It causes alterations in growth and behavior, renal function deficits, hypertension, osteoporosis, lead-induced anemia; affects a wide range of physiological systems and organs, including the central nervous system, the cardiovascular system, the gastrointestinal tract etc. [1–4]. Lead affects children health [5]. Lead is a significant genotoxic agent [6–8]. Lead produces an excessive amount of reactive oxygen species resulting in oxidative stress and chronic kidney disease [9]. This metal has also multiple hematological effects. Microcytosis and hypohromy of red blood cells appear because of

disturbed heme synthesis [10]. Significant decreases in red blood cells and mean corpuscular hemoglobin, and significant increase in the frequency of micronucleated polychromatic bone marrow erythrocytes were found in Algerian mice (*Mus spretus*) exposed to Pb [11]. Lead damages the chromosomal structure in mammalian cells [12–16]. At toxic doses, lead acetate and lead nitrate have induced DNA breaks determined by nick translation [17].

Therefore, the efforts for Pb neutralization in the animal organism are of great importance. A significant part of lead (Pb^{2+}) intake could be neutralized in the stomach. For that purpose, zeolites, which reveal a unique selective adsorption and structural stability under high temperatures and acidity, are considered as a reliable means [18–19]. According to EMFEMA (2005), zeolites also allow better performance of intestinal microflora [20].

Clinoptilolites are crystalline, hydrated aluminosilicates of alkali and alkaline earth cations, consisting of three-dimensional frameworks of SiO_4^{4-} and AlO_4^{5-} tetrahedra linked through the shared oxygen atoms [21]. They are the most abundant natural zeolites [22], occurring in volcanic and sedimentary rocks. Their molar Si/Al ratio is above 4 [23–24]. The structure of clinoptilolites is characterized by large intersecting open channels of 10- and 8-member tetrahedral rings [25]. The total pore volume of approximately 35% [26], and chemical formula (K, Na, Ca, Mg).($AlSi_{43}O_8$).$5H_2O$ [27]. Such structure ensures the clinoptilolite capacity of to adsorb and accumulate heavy metals [28–32]. The clinoptilolites are a perfect heavy metal-trap because of the fact, that Si-block is neutral, while the Al-block in crystalline unit is negative, and thus it charges the mineral's lattice negatively. The existence of Na, K and/or Ca cations determines the neutrality of the minerals. These cations are exchanged in solutions with cations of certain metals, such as Pb, Cd, Hg, etc. [22, 33]. The effects of clinoptilolites in animals appear to be related to their high cation-exchange capacity, which affects tissue uptake and utilization of NH^{4+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cs^{+}, and other cations [32, 33]. Clinoptilolites appears to be stable in the gastrointestinal tract [34]. Therefore, the harmful lead ions (Pb^{2+}) could be trapped in the stomach. The high affinity of clinoptilolite for Pb would significantly reduce the amount of dietary Pb available for absorption by the intestinal mucosa.

The effects of reduction of Pb load, resulting from the feature of modified clinoptilolite KLS-10-MA to absorb Pb in gastrointestinal tract of mice, were discussed in detail by Beltcheva et al. [35]. In this study chromosomal aberrations and mitotic index, erythrocyte morphology, and body weight gain are considered as suitable biomarkers for assessment the detoxification

effect of the modified clinoptilolite sorbent KLS-10-MA administrated as a food supplement to ICR laboratory mice chronically exposed to Pb.

Other aspect is also in the scope. Many authors report the positive role of zeolites in livestock breading [36–40]. Thus, one of the aims of the present work is to prove whether a significant change in body weight would be observed in mice supplemented with clinoptilolite. For this reason, and in order to examine the reaction of mammal's organism to the sorbent, an additional control was used – healthy animals non-exposed to Pb and fed with conventional forage mixed with KLS-10-MA.

It is interesting to investigate quantitatively the response of the animal organism to heavy metals load as well as to such a load combined with antidote therapy. Here a mathematical model is proposed to outline the common trends of the kinetics of the lead bioaccumulation in the bones of the mice – exposed and non-supplemented and exposed and sorbent-supplemented. The model allows calculate some parameters.

For the first time a mathematical model for the change of mitotic index with time is proposed. On the base of this model the main trends of the behavior of the mouse's genetic apparatus, in conditions of a chronic metal intoxication, are explained.

2. Experimental settings

2.1 General

We have conducted the experiment in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes and according to the current Bulgarian laws and regulations.

The ecotoxicological experiment covered 90 days. The samples for the investigations were taken in days 15, 45, 60 and 90 of the exposure. At each time point, a subset of eight mice from the four groups was used.

2.2 Animals

Laboratory mice, inbred ICR strain, only males, about 8 weeks of age, were used. The animals were arranged in four groups each of 60 specimens, as follows: *Group 1*, (control) animals fed with conventional food for small rodents and water; *Group 2*, animals fed with conventional food + clinosorbent KLS-10-MA and water; *Group 3*, animals fed with conventional food and

water + $Pb(NO_3)_2$; *Group 4*, animals fed with conventional food + sorbent KLS-10-MA and water + $Pb(NO_3)_2$.

All animals were bread in vivarium and housed in individually ventilated cages. The physical size of the cages was in accordance with European standards. The bedding material was obtained from an ISO 2000 accredited supplier. Mice were acclimatized for a 7-day period before starting the experiment. A standard temperature of between 19-23°C, a humidity of 45-60 % and a 12-hour light/night cycle were kept all the time. The food was in the form of pellets and not withheld at any time during the experiment. All mice were allowed access to food and water *ad libitum*. The animals were neither medicated nor vaccinated.

2.3. Sorbent preparation

The modified clinoptilolite was prepared by Nikolay Popov through a treatment of natural Bulgarian clinoptilolite (zeolite containing 82% clinoptilolite) obtained from the region of East Rhodops in South Bulgaria. The natural clinoptilolite was heat-treated at 240-250^{o}C and then chemically-mechanically activated with 10% alkaline salt, addition of 25-weight % distilled water, and 4 hours processing in ball-crusher (wet activation). Chemical composition of the clinoptilolite sorbent KLS–10-MA was determined by common analytical method for silicate materials. Cations exchange capacity was determined according to the method of Ming ad Dixon [41]. A value of 5.98 has the Greek natural clinoptilolite successfully used for treatment of solutions containing Pb^{2+}, Cu^{2+} and Zn^{2+} [22].

The chemical compositions of the natural and modified clinoptilolites are given in Table 1.

Clinoptilolite chemical composition %		
Constituent	Natural	KLS-10-MA
SiO_2	69.3	66.09
Al_2O_3	11.6	10.62
Fe_2O_3	1.7	0.76
TiO_2	0.1	0.12
CaO	2.11	3.03
MgO	2.37	0.33
Na_2O	0.98	4.49
K_2O	1.59	3.11
I. L.	10.32	11.37
Molar Si/Al	5.97	6.22

Table 1. Chemical composition (%) of clinoptilolite samples: natural (Golobradovo, South-East Rhodops Mountain, Bulgaria) and modified (KLS-10-MA)

The ratio SiO_2/Al_2O_3 in zeolites is a marker of great importance to determine their acid stability. A greater SiO_2/Al_2O_3 ratio correlates with higher acid stability of the zeolites [34]. Thus, the specific structure and high SiO_2/Al_2O_3 ratio in clinoptilolite makes it the preferable sorbent in different acid solutions, including gastric juice. Tao et al. investigating Na-clinoptilolite established that it maintained structure stability in solution with pH value of 1.2 [25], while Zhou and Zhu indicated a premature collapse of zeolites NaY and NaA in such strong acid solution [30].

The most important feature of zeolites, which determines their wide usefulness, is their cation exchangeability [31]. The aluminum ion is small enough to occupy the position in the center of the tetrahedron of four oxygen atoms, and the isomorphous replacement of Al^{3+} for Si^{4+} raises a negative charge in the lattice [33]. The negatively charged framework counter-balanced by positive cations (Na, K and Ca), results in a strong electrostatic field on the internal surface. These cations can be exchanged to fine-tune the pore size or the adsorption characteristics. All clinoptilolite modifications are based on these principles. The pore size has an essential function in the Na^+, K^+, or Ca^{++} exchange with certain cations such as Pb^{2+}, Cd^{2+}, Zn^{2+} etc. in solutions.

Samples	Natural	KLS-10- MA
Cation exchange capacity	102	139.5
Exchangeable cations		
Na^+	23.34	133.14
K^+	19.14	36.08
Ca^+	61.22	24.68
Mg^+	1.05	2.41
Total	104.75	196.31

Table 2. Cation exchange capacity (meq/100g, NH_4^+) and exchangeable cations (meq/100g) of clinoptilolite samples from Golobradovo, South-East Rhodops Mountain, Bulgaria (natural and modified)

The sorbent KLS-10-MA used in the present experiment is a Na-enriched sorbent [35]. The cation exchange capacity of KLS-10-MA was almost 1.37-fold higher compared to that of the natural clinoptilolite (Table 2). The exchangeable sodium cations in KLS-10- MA were 5.7-fold more and the total exchangeable cations were 1.9-fold more than those in the natural matter.

Results of several authors indicate that Na-enriched form of modified clinoptilolite has highest static ion exchange ability towards Pb^{2+}, Cd^{2+}, NH_4^+ etc. [8, 42]. Thus, the modification KLS-10-MA could be considered as a successful sorbent for detoxification purposes.

2.4. Treatment

Animals were arranged in four groups each of 60 specimens, as follows: Group 1 (Control) , animals fed with conventional food for small rodents and water; 2) Group 2, animals fed with conventional food + clinosorbent KLS-10-MA and water; 3) Group 3, animals fed with conventional food and water + $Pb(NO_3)_2$; 4) Group 4, animals fed with conventional food + KLS-10-MAand water + $Pb(NO_3)_2$.

The exposure to Pb was performed as the mice were treated with 0.05 N solution of lead nitrate ($Pb(NO_3)_2$), diluted 1:10 in the drinking water.

The clinoptilolite sorbent KLS–10-MA, as a powder, was mechanically mixed at 12.5% concentration with the conventional forage for small rodents.

2.5. Lead concentrations determining

The concentrations of Pb in the whole body, liver, kidney, bones, and feces of the control and experimental animals were determined on days 15, 40, 60, and 90 from the beginning of the experiment.

To determine the Pb concentration, after the removal of the alimentary tract, the tissues and some internal organs were oven dried at 60^0 C to a constant weight. The dried tissues were dissolved in a mixture of concentrated nitric–perhloric acid (4:1) [43]. The concentrations of Pb and the element composition of the two food variants were determined in a certified laboratory by atomic emission spectrometry with inductively coupled plasma (ICP AES) on a GFAAS–Varian instrument. The detection limits were 0.002 mg/l for Mn; 0.004 mg/l for Cd; 0.005 mg/l for Zn; 0.03 mg/l for Pb; 0.04 mg/l for Fe; 0.5 mg/l for Ca, K, Mg, and Na.

2.6. Cytogenetical analysis

Aberrant mitoses (in percentages) were determined as described by Preston et al. [44]. Mitomycin C (3.5 mg/kg) (Fluka) was used as a positive control. The other animals were injected with only 0.2 mL 0.9% NaCl. Bone marrow chromosomal aberration assays were performed in groups of animals, each one consisting of 8 specimens. The animals were injected ip with colchicine at a dose of 40 mg/kg, 1 h before isolation of bone marrow cells. The bone

marrow cells were flushed from femur with 0.075 M KCl and hypotonized at 37^0C for 20 min. Thereafter the cells were fixed in methanol-acetic acid (3:1), dropped onto cold slides, and air dried. To examine the chromosomal aberrations the slides were stained with 5% Giemsa solution (Sigma Diagnostic). At least 50 well-spread metaphases were analyzed per animal at random.

The mitotic indices were determined by counting the number of dividing cells among 1500 cells per animal. The frequencies of abnormalities and the mitotic index were determined for each animal. The mean ±SD for each group was calculated and the data were statistically evaluated for their significance by analysis of variance using Student *t* test.

2.7 Hematological analysis

The hematological analysis was carried out on the same groups of animals using standard clinical methods. Peripheral blood samples were collected between 9 and 11AM from the orbital sinus [45]. The percentages various form of cells were determined using Giemsa stains. About 150-200 cells were counted in each stain.

2.8. Statistical analysis

Statistical analysis was done by using the SPSS Package for Windows, version 15.0. Differences were considered to be significant when *p* values were lower than 0.05 ($p < 0.05$). First, the data were processed according to the Kolmogorov-Smirnov test for normality in each group. All groups showed normal distributions and the data were then analyzed by Analysis of Variance and subsequent Tukey HSD test (High Statistical Difference) and Dunnet test, for estimating individual differences.

3. Mathematical model for lead bioaccumulation

This mathematical model describes the process of the lead bioaccumulation in animals´ bones. Three "compartments" of Pb movement are considered: gastrointestinal tract, blood and bones. One can assume that Pb is distributed evenly into compartments, which allows the use of differential equation for its kinetics. After entering in gastrointestinal tract, Pb moves to blood and then to bones. Thus, the following system of ordinary differential equations takes place:

$$\frac{dx}{dt} = -a_1 x - a_2 x \qquad (1)$$

$$\frac{dy}{dt} = a_1 x - a_3 y - a_4 y \qquad (2)$$

$$\frac{dz}{dt} = a_3 y \quad (3)$$

Under initial conditions:

$$t_0 = 0, \quad x(t_0) = x_0 = A, \; y(t_0) = 0, \; z(t_0) = z_0 \quad (4)$$

where x, y, and z are the concentrations [mg/kg] of Pb in the gastrointestinal tract, blood, and bones, respectively; t is time [days]; t_0 is the moment when the experiment starts; a_1 ($[a_1] = [\text{day}^{-1}]$) and a_3 ($[a_3] = [\text{day}^{-1}]$) are the rate constants of Pb accumulation in blood and bones, respectively; a_2 ($[a_2] = [\text{day}^{-1}]$) and a_4 ($[a_4] = [\text{day}^{-1}]$) are the rate constants of Pb excretion through the feces and urine, respectively; *dx/dt, dy/dt,* and *dz/dt* are the rates of change in Pb levels in the three compartments, respectively.

For the equation (3) under conditions (4), the following analytical solution was obtained:

$$z(t) = z_0 + Aa_1a_3 \left(\frac{1}{b_1b_2} - \frac{1}{b_1(b_2 - b_1)}e^{-b_1t} + \frac{1}{b_2(b_2 - b_1)}e^{-b_2t}\right) \quad (5)$$

where $b_1 = a_1 + a_2$ and $b_2 = a_3 + a_4$.

The solution $z(t)$, representing the process of Pb bioaccumulation in the bones of the mice, is graphically given in Figure 1. The initial condition $z(t_0) = z_0$ corresponds to the bone Pb concentration in the Control group, $z_0 = 1$ mg/kg. The concentration of Pb in the drinking water of the experimental animals was 620 mg/l ≈ 620 mg/kg. The daily water consumption per animal was about 7 ml/day and therefore, the daily Pb dose per animal could be approximately $B = 4.34$ mg/day. Extrapolating over the experiment, and taking into account the value of gastrointestinal resorption coefficient $\eta = 15\%$ [46], it was calculated that in Group 3 the entire quantity of Pb absorbed by the gastrointestinal mucosa and entering into the blood during the experiment might be $A_{\text{Group3}} = 58.6$ mg. Coefficient η ($0 \le \eta \le 1$) is a dimensionless coefficient indicating what fraction of ingested metal dose resorbs in the digestive tract. Converted to concentration in mg/kg, and taking into account that the mean mouse body weight during the experiment was about 30 g, we obtained $A_{\text{Group3}} = x\,(t_0) = 1953$ mg/kg. The parameters were fitted by minimization of χ^2 by the use of an iterative Gauss-Newton procedure [47, 48]. Thus, the following values were found – for Group 3: $a_1 = 0.022$ day^{-1}, $a_2 = 0.001$ day^{-1}, $a_3 = 0.099$ day^{-1}, and $a_4 = 0.004$ day^{-1}; for Group 4: $A_{\text{Group4}} = x\,(t_0) = 459.6$ mg/kg, $a_1 = 0.022$day^{-1}, $a_2 = 0.002$ day^{-1}, $a_3 = 0.099$ day^{-1}, and $a_4 = 0.004$ day^{-1}. Based on the value A_{Group4}, absorption coefficient η =

3.53% was calculated for Group 4 (versus η = 15% in non-supplemented mice) by the formula:

$$\hbar = \frac{A_{\mathrm{Group}_4} P}{1000BT}.$$

P is the mean mouse body weight during the experiment, B is the daily dose of Pb per animal, and T is the duration of the experiment. The model shows that the rate constants of Pb excretion by the feces $a_{2\mathrm{Group}\ 4}$ is 2-fold higher than $a_{2\mathrm{Group3}}$. This is in correspondence with the real situation. The clinoptilolite sorbent accelerates the intestine passage, as a ballast matter; therefore the Pb elimination is more intensive in Group 4.

The value η = 3.53% obtained in clinoptilolite supplemented mice (Group 4) is 4.25-fold lower compared with η = 15% in unsupplemented animals. A reduction of 76% occurred! This is a significant result: KLS-10-MA diminished the Pb absorption in gastrointestinal tract of mammals´ organism more than four times.

4. Mathematical model for mitotic index

The behavior mode of the mitotic index (MI) during the experiment suggests that a quantitative approach would be of great benefit.

The change of MI over time results from two main processes: 1) decrease of MI due to the toxic effect of Pb and 2) increase of MI due to recovery processes running in the mouse's organism.

In unsupplemented animals the recovery process is quite week, so there only a drop of MI was observed, although this drop was going with a decreasing rate. In this case the following differential equation is adequate:

$$\frac{dm}{dt} = -a(t)m \qquad (6)$$

under initial condition:

$$t_0 = 0, \quad m(t_0) = m_0 \qquad (7)$$

where m (%) is the value of MI (the percentage of cells undergoing mitosis), dm/dt is the rate of change of MI with the time, $a(t)$ ($[a] = [\mathrm{day}^{-1}]$) is sensitivity (a parameter, characterizing the cell sensitivity to Pb toxicity, expressed as a "rate constant" of the diminution of the cells with proliferative activity. This parameter is presumed as a time dependent variable because the experimental data clearly show a decreasing intensity of the cell reaction to Pb bioaccumulation,

probably due to the certain detoxification of the organism on the basis of liver and kidney activity in the course of the Pb treatment. Thus, the sensitivity of the bone marrow cells to Pb decreases during the experiment. Therefore, the parameter $a(t)$ decreases with the time.

At simplest $a(t)$ cold be modeled according to the following mechanism:

$$\frac{da}{dt} = -ra \qquad (8)$$

under initial condition:

$$t_0 = 0, \quad a(t_0) = a_0 \qquad (9)$$

where r ($[r] = [\text{day}^{-1}]$) could be denoted as reduction parameter (reduction of the sensitivity regarding Pb toxicity). The solution of the differential equation (8) under initial condition (9) is:

$$a(t_0) = a_0 e^{-rt} \qquad (10)$$

Taking into account (10), the differential equation (6) could be written in the form:

$$\frac{dm}{dt} = -a_0 e^{-rt} m \qquad (11)$$

The solution of the equation (11) under initial condition (7) is:

$$m = -m_0 e^{-\frac{a_0}{r}(1-e^{-rt})} \qquad (12)$$

The initial condition is $m(t_0) = m_0 = 12\%$.

In the supplemented animals, the recovery processes were appreciable and more intensive because the clinoptilolite adsorbs a significant part of Pb in the digestive tract. After day 45 the mitotic index significantly increased. Besides, our results showed that MI in clinoptilolite–supplemented mice remains the entire time on visible higher levels compared with that in unsupplemented ones (Figure 2). The following differential equation is adequate to describe MI behavior in the case of clinoptilolite supplementation:

$$\frac{dm}{dt} = -a_0 e^{-rt} m + k(t) \qquad (13)$$

under initial condition:

$$t_0 = 0, \quad m(t_0) = m_0 \qquad (14)$$

The parameter k ($[k] = [\%\ \text{day}^{-1}]$) could be named *recovery rate* and it could be considered as an integral characteristics of the whole complex of recovery processes developing in the animal organism. These processes, accelerated by clinoptilolite supplementation, run in parallel with the injuries, caused by Pb intoxication. The experimental data suggest that parameter k is a variable

quantity. It seems reasonable to propose the following differential equation for the time course of k:

$$\frac{dk}{dt} = g - ck \qquad (15)$$

under initial conditions:

$$t_0 = 0, \qquad k(t_0) = k_0 \qquad (16)$$

The parameter g ([g] = [day^{-2}]) is presumed as a genetically determined parameter and it could be considered as an integral characteristic of the recovery potential of the genetic apparatus. The parameter c ([c] = [day^{-1}]) plays a role of a restriction constant related to the inhibition of the recovery process due to the toxicant.

The solution of the differential equation (15) under initial condition (16) is:

$$k = k_0 e^{-ct} + \frac{g}{c}(1 - e^{-ct}) \qquad (17)$$

Taking into account Eq. (17), the differential equation (13) could be written in the form:

$$\frac{dm}{dt} = -a_0^{-rt} m + k_0 e^{-ct} + \frac{g}{c}(1 - e^{-ct}) \qquad (18)$$

The equation (18) cannot be solved analytically and numerical solution was obtained.

The time courses of the mitotic indices in groups 3 and 4, determined by the mathematical model, are graphically displayed in Figure 1. There, the experimental results are also presented.

5. Zeolites reduce lead bioaccumulation

The highest Pb concentrations were established in feces, followed by those in bones of the mice from Group 3. The background Pb levels in carcass, liver, kidney, bones, and feces of the control mice were 0.22 ± 0.06, 0.5 ± 0.07, 0.44 ± 0.08, 0.99 ± 0.09, and 23.6 ± 6.7 mg/kg, respectively.

On day 90 the Pb concentrations in Group 3 in carcass, liver, kidney, bones, and feces were 1467-fold, 133-fold, 1337-fold, 1523-fold, and 406-fold higher compared to those in the Control group.

The treatment with the clinoptilolite sorbent essentially improved the status of the Pb-exposed mice. In Group 4, Pb concentrations in carcass, liver, kidney, bones, and feces were 237-fold, 17-fold, 125-fold, 357-fold, and 51-fold higher compared to the Control group.

The differences between Group 3 and Group 4 were quite significant ($p > 0.001$). The reduction in Pb levels in the exposed and supplemented mice compared to exposed unsupplemented ones was as follows: 84%, 89%, 91%, 77%, and 88% for carcass, liver, kidney, bones, and feces, respectively.

The ratio $Pb_{90}/Pb_{15} = R$ could be called *bioaccumulation coefficient*. The following ratios $R = Pb_{90}/Pb_{15}$ were calculated for carcass, liver, kidney, bones, and feces – in Group 3: $R_{Carcass} = 7.68$, $R_{Liver} = 7.89$, $R_{Kidney} = 3.85$, $R_{Bones} = 7.43$, $R_{Feces} = 2.85$; and in Group 4: $R_{Carcass} = 2.83$, $R_{Liver} = 0.7$, $R_{Kidney} = 1.02$, $R_{Bones} = 4.27$, $R_{Feces} = 1.3$, respectively.

When denote $(Pb_{90}/Pb_{15})_{Group3} = R_3$ and $(Pb_{90}/Pb_{15})_{Group4} = R_4$, the following relations take place:

$(R_3/R_4)_{Carcass} = 2.7$; $(R_3/R_4)_{Feces} = 2.19$ (19)

$(R_3/R_4)_{Liver} = 11.27$; $(R_3/R_4)_{Kidney} = 3.77$; $(R_3/R_4)_{Bone} = 1.74$ (20)

It is clear that the bioaccumulation coefficients in the exposed and unsupplemented mice are much higher than in the exposed and supplemented ones. The relations (19) and (20) exhibit the significant reduction of this coefficient in Group 4, especially for the liver. It is once again an evidence for the significant drop of Pb bioaccumulation caused by the ion exchange capacity of the sorbent KLS-10-MA. Thus, the high effectiveness of the clinoptilolite versus lead toxicity is confirmed.

The following ratios Pb_{90}/Pb_{15} were calculated for the carcass, liver, kidney and bones of the experimental animals – in Group 3: 7.68, 7.89, 3.85, 7.43 and in Group 4: 2.83, 0.7, 1.02, 4.27 respectively. As expected, the bioaccumulation coefficients for all studied organs and tissues are much higher in exposed and unsupplemented mice (Group 3) than in exposed and supplemented ones (Group 4). This result once again underlines the significant role of the ion exchange capacity of the sorbent KLS-10-MA for the reducing of Pb quantity entering the blood stream from the digestive tract.

To consider more accurately the bioaccumulation levels in the different tissues resulting from the sorbent supplementation, the following ratios were calculated for day 90:

Group 3 $Pb_{Bone}/Pb_{Liver} = 19$; $Pb_{Bone}/Pb_{Kidney} = 2.5$; $Pb_{Kidney}/Pb_{Liver} = 7.6$ (21a)

Group 4 $Pb_{Bone}/Pb_{Liver} = 40$; $Pb_{Bone}/Pb_{Kidney} = 6.4$; $Pb_{Kidney}/Pb_{Liver} = 6.3$ (21b)

The ratios (21a) and (21b) show the great differences between Pb-levels in bone and kidney, and especially between Pb-levels in bone and liver. In Group 4 bone/liver Pb ratio was 2.1-fold

higher and bone/kidney Pb ratio was 2.56-fold higher compared to group 3. The same expressions show that kidney/liver Pb-ratio was almost not influenced by the supplementation. (Obviously, it is related to a strong relationship between liver and kidney Pb levels). These relations clearly indicate that the clinoptilolite sorbent exerts a significant detoxification effect in the soft tissues.

Another ratio, calculated also for day 90, could help to estimate the significant decrease of Pb bioaccumulation in the conditions of the sorbent supplementation:

Bone	$Pb_{Group\ 3}/Pb_{Group\ 4} = 4.3$	(22a)
Liver	$Pb_{Group\ 3}/Pb_{Group\ 4} = 9$	(22b)
Kidney	$Pb_{Group\ 3}/Pb_{Group\ 4} = 11$	(22c)

The equations 22a, 22b, and 22c showed that the highest reduction of Pb bioaccumulation, due to the supplement, occurs in the kidney. This means that KLS-10-MA sharply decreases the Pb level in the blood. In fact, the sorbent acts in the gastrointestinal tract and significantly lowers the Pb-resorption by the mucosa. Thus, the present study confirms the assumptions of other authors that clinoptilolite sorbents can be reliable potential candidates for application in conditions of gastric juice [25].

The reduction of 88% of Pb content in the feces of the exposed and supplemented mice, compared to exposed and unsupplemented ones indicates that clinoptilolite supplementation can prevent, to some extent, the contamination of the environment.

6. Zeolites reduce injuries

6.1. Zeolites stimulate the genetic apparatus

The chromosome aberration frequency (CAF) in the analyzed metaphases of bone marrow cells of ICR mice is presented in Figure 3. The percentages aberrant metaphases in the Control group were within the range of spontaneous frequencies. No statistically significant differences ($p < 0.1$) were observed between CAF in groups 1 and 2 except on day 90 ($p < 0.05$). No statistically significant differences were observed between CAF in groups 4 and 2 except on day 60 ($p < 0.05$). Significant differences ($p < 0.001$) were recorded between Group 3 and other groups at each time points. CAF in Group 3 (CAF_3) exhibited on average 2-fold higher level compared to group 4 and 3.5-fold higher level compared to the Control group. On day 90 Pb-exposed and clinoptilolite-supplemented mice exhibited 2.3-fold lower chromosome aberrations

frequency and 2.5-fold higher mitotic index compared to the Pb-exposed and unsupplemented mice.

These results once again confirm the essential benefit of the clinoptilolite sorbent.

6.2. Zeolites improve the blood status

Lead bioaccumulation caused statistically significant reduction of the percentage normal erythrocytes and respectively significant increase of the percentage pathological erythrocytes in the peripheral blood of the mice from Group 3, compared to the Control group ($p < 0.001$) (Figure 4). The normal and pathological erythrocytes decreased and increased, respectively, in Group 4, but the normal red blood cells were at significantly higher level and the pathological ones were at significantly lower level compared with Group 3 ($p < 0.01$). Besides, within Group 4 the normal erythrocytes remained significantly higher than pathological ones ($p < 0.001$) up to the end of experiment.

The following ratios were calculated between normal and pathological erythrocytes within groups 3 and 4, respectively on days 15 and 90:

Group 3: $N_3/P_3 = 1.9$ (15), $N_3/P_3 = 0.71$ (90) (23)

Group 4: $N_4/P_4 = 3.1$ (15), $N_4/P_4 = 1.3$ (90) (24)

Between groups 3 and 4, and the Control group, on day 90, the following ratios were established regarding the normal and pathological erythrocytes, respectively:

$N_1/N_3 = 2$, $N_1/N_4 = 1.35$ (25)

$P_3/P_1 = 2.3$, $P_4/P_1 = 1.9$ (26)

These results show that the clinoptilolite sorbent diminishes injuries in the blood of the treated animals.

6.3. Zeolites co operate for the body weight gain enhancement

Statistically significant differences were obtained among the body weight (W) in the different groups ($p < 0.01$) on days 15, 45, 60 and 90 (Figure 5). A sharp decrease of body weight was established in the Pb-exposed mice from Group 3 after day 60. These mice exhibited a body weight gain reduced with 24% compared to the control mice (calculated on day 90). The body weight gain in the exposed and supplemented mice was 21% higher compared to that in the exposed and unsupplemented ones. No statistically significant difference was found between Group 4 and Control group during the experiment. These groups showed a higher body weight in relation to Group 3. A weak acceleration of body weight gain was established in the conditions

of clinoptilolite supplementation: a 5% higher body weight was recorded in the healthy supplemented mice (Group 2) compared with the control ones (Group 1). Thus, the mice from Group 2 (healthy animals supplemented with clinoptilolite sorbent KLS-10-MA) showed the highest body weight compared with other groups.

Conclusion

The modified natural clinoptilolite sorbent KLS-10-MA, a Na-enriched alkali earth clinoptilolite, based on natural Bulgarian clinoptilolite, strongly decreased the absorption of lead in animals´ gastrointestinal tract and thus limited Pb quantity entering the blood. The mice exposed to Pb and supplemented with KLS-10-MA exhibited a reduction in Pb levels in several samples of about 77 – 90%. The relations Pb_{90}/Pb_{15} for carcass, liver, kidney, bones, and feces in supplemented animals were significantly lower compared to those in unsupplemented ones. The chromosome aberrations frequency decreased by 2.3 times, and the mitotic index increased by 2.5 times in the clinoptilolite supplemented mice. The proportion between normal and pathological erythrocytes in the clinoptilolite-supplemented mice was 1.83-fold higher than this in unsupplemented animals. A weak rise of the body weight gain was established in the healthy clinoptilolite-supplemented animals**.** No toxic effects of the clinoptilolite sorbent on the experimental animals were observed.

The mathematical model of Pb bioaccumulation in bones is in accord with the experimental data and well predicts the time course of Pb concentrations in conditions of chronic exposure to Pb with/without sorbent supplementation. On the base of this model gastrointestinal resorption coefficient η for Pb was calculated. In the exposed and clinoptilolite-supplemented mice, η = 3.53%, was 4.25-fold lower compared with η = 15% in unsupplemented mice.

The mathematical model for the change of the mitotic index helps to understand that recovery processes in the animals run in parallel with the Pb bioaccumulation and that the susceptibility of the mouse's organism to Pb load decreases and the recovery rate of the genetic apparatus increases during the experiment. The model helps made a quantitative evaluation of the positive effect of the clinoptilolite treatment. Such models could benefit the toxicological investigations.

Because of the significant favorable effect of the clinoptilolite sorbent KLS-10-MA it could be recommended as a reliable tool for detoxification of human and animal organisms chronically

poisoned by heavy metals, particularly lead. The results of this study encourage a further elaboration of a reliable drug based on the tested substance. A drug based on the clinoptilolite sorbent could be very useful for a supplementation of animals in regions that are industrially polluted with heavy metals, and particularly with Pb, in order to protect the animals´ health and the quality of the environment.

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Caption of figures

Figure 1. Lead bioaccumulation in bones of ICR mice from groups 3 and 4 during the conducted ecotoxicological experiment: model solutions and experimental points. The time point "*0*" corresponds to the concentrations in Control group

Figure 2. Time course of the mitotic indices, during the experiment, in the four groups of the studied ICR mice. The results were recorded at 15^{th}, 45^{th}, 60^{th} and 90^{th} days of treatment.

Figure 3. Time course of the chromosome aberrations frequency in ICR mice – exposed to Pb (Group 3) and exposed to Pb and clinoptilolite-supplemented (Group 4). The control group (Group 1) and the second control (healthy and supplemented mice) (Group 2) are also presented

Figure 4. Percentage of the normal and pathologically changed erythrocytes, during the experiment, in the four studied groups of ICR mice

Figure 5. Time course of the mean body weight of investigated ICR mice, in the four groups, during the experiment