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Zeolites Significantly Diminish Lead Intoxication Harm

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ABSTRACT

The purpose of the present article is to outline the high effective properties of the clinoptilolite matter as a sorbent of toxic substances, especially lead (Pb), in living organisms. For the first time, the act of zeolite as an absorbent, in conditions of Pb intoxication in living organism was proved. A clinoptilolite sorbent was prepared based on natural Bulgarian zeolite and applied in a 90 day ecotoxicological experiment. The dietary inclusion of the sorbent reduced the lead concentrations in the Pb exposed and clinoptilolite-supplemented mice by 84%, 89%, 91%, 77% and 88% in carcass, liver, kidneys, bones and feces, respectively. Significant improvement of the chromosome aberrations, mitotic index and erythrocyte morphology and erythropoiesis and body weight was established in the clinoptilolite-treated animals.

A mathematical model for lead concentration was elaborated and thus, the coefficient (η) of Pb absorption by gastrointestinal mucosa in the supplemented mice was found. It is: η=3.53% (versus η=15% in non-supplemented ones).

For the first time a mathematical model for the mitotic index was proposed. It shows that the animals' recovery goes in parallel with the Pb bioaccumulation and the susceptibility of the mouse's organism to Pb load decreases, while the recovery rate of the genetic apparatus increases with the time.

Keywords: Zeolite, Clinoptilolite, Lead intoxication, Chromosome aberration, Mitotic index, Mathematical models, Absorption coefficient

INTRODUCTION

Zeolites are a large, widespread family of minerals. Their physical and chemical properties make them suitable means for different areas of applications [1,2]. Because of their specific structure, they are excellent absorbents and thus-an excellent means against chronic heavy metals intoxication and particularly lead intoxication. These minerals are intensively used for augmentation of animal production, growth performance, feed utilization [3] and prevention of certain farm animal diseases [4] as well as for the protection of the environment [5-7]. Zeolites are applied in biotechnology and medicine [8]. They are very useful in processes of the removal of ammonium [9].

Clinoptilolite is a mineral from the zeolite group. It is known as one of the most excellent toxin absorbents. It is the most widely used zeolite in animal studies due to its structural stability under high temperatures and acidity.

For the first time we proved the effect of zeolite, as absorbent, in conditions of Pb intoxication of a living organism. A modified clinoptilolite sorbent, named "KLS-10-MA", water modification (made in Bulgaria, by Dr. Nikolay Popov), was applied, as a food supplement, in a 90-day ecotoxicological experiment with laboratory mice inbred line ICR.

Here, the morphophysiological and cytogenetical parameters, indicting the physiological status of a living organism, are designated as "biomarkers".

One of the most important of them is the chromosomal aberration frequency (CAF) [10,11]. Along the increase of CAF, the decrease of the proliferation capacity of the cells, indicated by mitotic index (MI), is used as indicator (biomarker) of different disorders in the genetic apparatus.

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Especially useful are CAF and MI for the assessment of the environmental contamination degree [12-14].

A mathematical model of the kinetics of lead bioaccumulation in bones in the exposed and exposed-supplemented animals was constructed (it is presented in the respective section). For the first time a mathematical model, presenting the common trends of MI change in conditions of a chronic intoxication was elaborated.

Because of their specific, well-defined pore structure [2], zeolites are extensively used in primarily three fields of application: adsorption, catalysis and ion exchange. In addition, natural zeolites because of their lower cost are used in bulk mineral applications. Zeolites are already in use in the food industry. According to EMFEMA [15], zeolites allow better performance of intestinal micro flora.

The mineral clinoptilolite (K, Na, Ca, Mg $(AlSi_3O_8).5H_2O$) belongs to Clinoptilolite-Heulandite group [16]. Clinoptilolite is a Heulandite which is reach of silica (silicon dioxide) SiO_2. (Heulandite is named in honor of the English collector of minerals John Henry Heuland). The name "clinoptilolite" is derived from the Greek words klino (κλίνω; "oblique"), ptylon (φτερών; "feather") and lithos (λίθος; "stone"). Really the texture of the clinoptilolite patterns resembles in some degree a feather. Clinoptilolite is a natural zeolite comprising a micro porous arrangement of silica and alumina tetrahedra.

Clinoptilolites are minerals with specific crystalline lattice-hydrated aluminosilicates of alkali and alkaline earth cations, consisting of three-dimensional frameworks of SiO_{44}- and AlO_{45}-tetrahedra linked through the shared oxygen atoms [4]. They have a relatively open structure with a total pore volume of approximately 35%.

The great benefit of the clinoptilolite as absorbent and particularly perfect heavy metal-trap is founded on the character of its structure. The Si-block in crystalline unit is neutral, while the Al-block is negative, 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 easily exchange in solutions with cations of certain metals, such as Pb, Cd, Hg, etc. [17-19] established that clinoptilolite exhibited the highest capability in absorbing Pb^{2+} ion in complex solution with pH value of 1.2 at 37°C, achieving the capacity of 7 mg/g, two times more than that by other zeolites and six times over that by activated carbon.

The effects of clinoptilolites in animals appear to be related to their high cation-exchange capacity [20], which affects tissue uptake and utilization of NH^{4+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cs^{+} and other cations [18,21].

Clinoptilolites support the immune activity [22-24]. They appear to be stable in the gastrointestinal tract [25] and as unique selective absorbents, they could absorb toxins, heavy metals and free radicals from the body and excretes [4,26]. Therefore, the harmful lead ions (Pb^{2+}) could be trapped in the stomach. It is believed that the high affinity of clinoptilolite for Pb would significantly reduce the amount of dietary Pb available for absorption by the intestinal mucosa [19].

Clinoptilolites of high quality occur in Bulgaria. These minerals have the most widespread distribution in the eastern and north-eastern Rhodopes Mountain (Rhodopes Mountain is situated in southwestern Bulgaria [27-29].

Clinoptilolite is practically a non-toxic substance [14]. A lack of toxic effects of clinoptilolite has been reported by other authors also [22,30-32].

MATERIALS AND METHODS

To explore in detail the positive effect of the clinoptilolite sorbent as absorbent of toxic substances, we conducted an experiment with laboratory mice, inbred ICR line. The experiment covered 90 days.

EXPERIMENTAL ANIMALS

Albino male mice, about 6-8 weeks of age, inbred ICR strain, were selected. The animals were arranged in four groups each of 60 mice:

1. 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 (as a powder) 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-MA sorbent and water+$Pb(NO_3)_2$

(Group 2 was a second control in order to prove eventual toxicity of the clinoptilolite sorbent)

The mice were bred 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 h 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 water, food and bedding material were daily inspected and changed when necessary. The animals were neither medicated nor vaccinated.

CLINOPTILOLITE

For the preparation of the modified clinoptilolite sorbent KLS-10-MA, natural clinoptilolite obtained from the deposit in the village Golobradovo (situated in the region of East Rhodops in South Bulgaria) was used. The sorbent was

prepared by Nikolay Popov through a treatment of natural clinoptilolite (zeolite containing 83.5% clinoptilolite) [33].

The modification KLS-10-MA could be considered as a successful sorbent for detoxification purposes. The lead resorption through the intestinal mucosa is prevented in a great extent.

STUDY DESIGN

The mice were chronically exposed to lead (Pb) in the form of aqueous solution of $Pb(NO_3)_2$, diluted in the drinking water. The modified clinoptilolite sorbent KLS-10-MA, in the form of powder mixed with the meal was used as antidote. The bioaccumulations of Pb and the detoxification effect of KLS-10-MA in carcass, organs and tissues, chromosomal aberrations and mitotic index, erythrocyte morphology and proliferation and body weight gain of the laboratory mice were studied.

The exposure of the mice to the lead was performed as the mice were treated with 0.05 N solution of lead nitrate diluted 1:10 in the drinking water. The clinoptilolite sorbent KLS-10-MA in the form of powder was mechanically mixed to a 12.5% concentration with conventional granulated forage for small rodents. Two variants of the feeding stuff [14] were prepared:

1. Standard (conventional) food [12]

2. Standard food mixed with 12.5% sorbent KLS-10-MA.

The concentrations of Pb in the whole body, liver, kidney, bones and feces and the percentages aberrant mitoses, mitotic indices, blood samples and body weight of the control and experimental animals were determined on days 15, 40, 60 and 90 [34] from the beginning of the experiment. At each time point a subset of 8 mice from the four groups were used.

To determine the Pb concentration, after the removal of the alimentary tract, the tissues and some internal organs were oven dried at 60°C to a constant weight. The dried tissues were dissolved in a mixture of concentrated nitric-perhloric acid (4:1) [35]. 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.

STATISTICAL ANALYSIS

The statistical analysis was done using the SPSS package for Windows, version 15.0. Differences were considered to be significant when p values were lower than 0.05 ($p<0.05$). Firstly, the data was processed according to Kolmogorov-Smirnov test for normality in each group. All groups showed normal distributions. Secondly, the data was analyzed by analysis of variance and subsequent Tukey high statistical difference test (Tukey HSD test) and Dunnet test, for estimating individual differences.

MATHEMATICAL MODEL FOR LEAD BIOACCUMULATION

This model helps calculate some kinetic parameters characterizing the Pb-accumulation and the degree of the sorption effect of the investigated mineral sorbent.

There are not enough studies that suggest quantitative approach. Physiologically based kinetic models (PBKM) for arsenic, chromium, mercury and lead have been proposed with any degree of completeness [36]. A simple mathematical model for Pb concentration in the body of mice, fed with a contaminated diet, was proposed. Series of PBKM for bone seeking elements have been developed [36]. Distribution and bioavailability of lead, chromium and uranium have been considered [37]. For the first time a mathematical model, describing the time courses of cadmium and zinc concentrations in liver and kidney of laboratory mice Mus musculus alba was proposed and it well explains the observed peculiarities of Cd bioaccumulation pattern in liver and kidney in conditions of very high exposure to heavy metal mixture [34].

In the present mathematical model for the kinetics of the lead bioaccumulation in mice's bones, three compartments of Pb movement are considered: gastrointestinal tract, blood and bones **(Figure 1)**. It was supposed that Pb is evenly distributed into these compartments. Entering in the gastrointestinal tract, Pb moves then to the blood and afterwards to the bones.

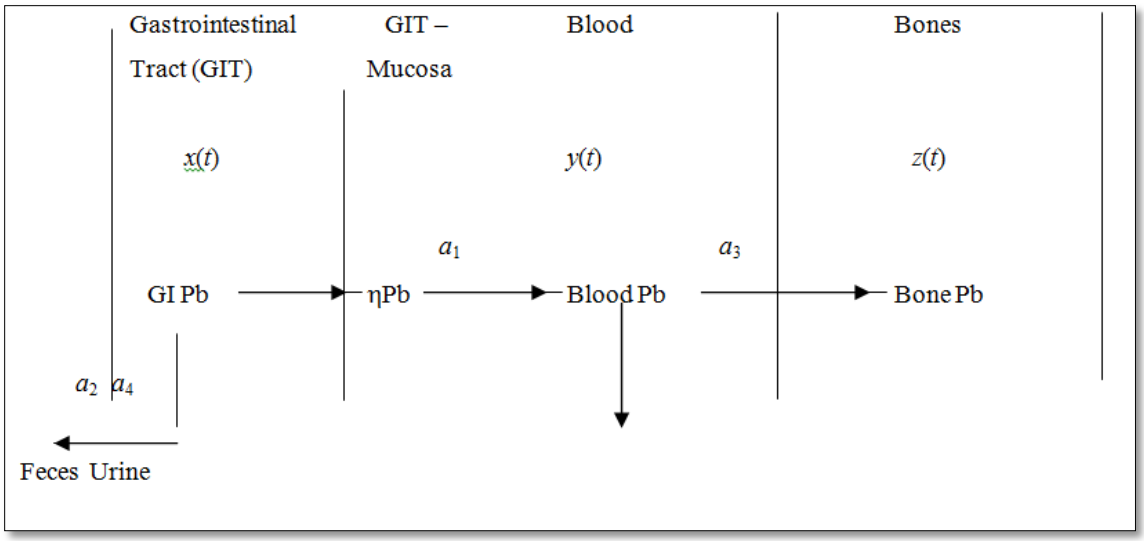

Figure 1. Scheme of the compartments, pathways and rate constants. The quantities x (t), y (t) and z (t) correspond to the Pb concentrations [mg/kg] (varying with the time t) in the gastrointestinal tract, blood and bones, respectively.

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:

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

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

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

Under initial conditions,

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

where t is time [days]; x, y and z are the concentrations [mg/kg] of Pb at a given time t in the gastrointestinal tract, the blood and the bones, respectively; t_0 is the moment when the experiment starts; $a1$ ([a_1]=[day^{-1}]) and a_3 ([a_3]=[day^{-1}]) are the rate constants of Pb accumulation in blood and bones, respectively; a_2 ([a_2]=[day^{-1}]) and a_4 ([a_4]=[day^{-1}]) are the rate constants of Pb excretion through the feces and the urine, respectively; dx/dt, dy/dt and dz/dt are the rates of the change in Pb levels with the time in the three compartments, respectively.

MATHEMATICAL MODEL FOR THE MITOTIC INDEX

The mathematical model, here presented, outlines the common trends in the change of the mitotic index over time, during a chronic Pb intoxication of small mammals. This change results from two main processes:

1. Decrease of MI due to the toxic effect of Pb
2. Increase of MI due to recovery processes running in the mouse's organism

In the 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 (Group 3):

$$\frac{dm}{dt} = -am \qquad (5)$$

Under initial condition,

$$t_0=0, \qquad m\ (t_0)=m_0 \qquad (6)$$

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]=[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 shows 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) should decrease with the time.

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

$$\frac{da}{dt} = -ra \qquad (7)$$

Under initial condition,

$$t_0=0,\ a\ (t_0)=a_0 \qquad (8)$$

where r ([r]=[day^{-1}]) could be denoted as reduction parameter (reduction of the sensitivity regarding Pb toxicity). The solution of the differential equation (7) under initial condition (8) is:

$$a(t) = a_0 e^{-rt} \qquad (9)$$

Taking into account (9), the differential equation (5) could be written in the form:

$$\frac{dm}{dt} = -a_0 e^{-rt} m \qquad (10)$$

In the supplemented animals the recovery processes are appreciable and more intensive because the clinoptilolite absorbs a significant part of Pb in the digestive tract. After day 45, MI significantly increased. It is known that natural clinoptilolite exerts an immunostimulatory effect [22]. Our results showed that MI in clinoptilolite-supplemented mice remains on a visible higher level compared with that in unsupplemented ones **(Figure 2)**.

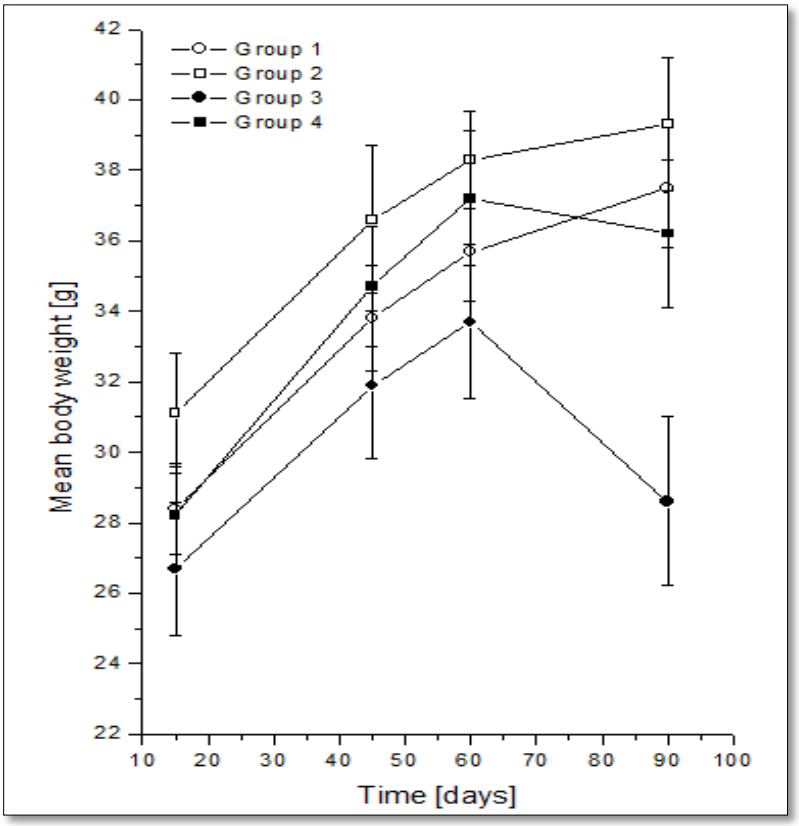

Figure 2. Changes of the mean body weight of the investigated ICR mice, in the four groups, during the experiment. It is seen, that in the unsupplemented mice, after day 60, the mean body weight strongly decreases because of the significant intoxication; contrariwise, in the intoxicated and supplemented animals, after day 60, the mean body weight remains almost without change; in the mice from Group 1 (control) and Group 2 (healthy and supplemented) the mean body weight increases with the time.

The following differential equation is adequate to describe MI behavior in the case of clinoptilolite supplementation (Group 4):

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

Under initial condition,

$$t_0=0, \qquad m\ (t_0)=m_0 \qquad (12)$$

The parameter k ([k]=[% day^{-1}]) could be named *recovery rate* and it could be considered as an integral characteristics of the complex of recovery processes developing in 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 the recovery rate k:

$$\frac{dk}{dt} = g - ck \qquad (13)$$

Under initial conditions,

$$t_0=0, \qquad k\,(t_0)=k_0 \qquad (14)$$

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 by the toxicant.

RESULTS AND DISCUSSION

Pb concentrations

Lead is significant genotoxic agent [38]. It is well studied as a factor damaging the chromosome structure in mammalian cells [12,13,39-41]. Our results are a new contribution to the previous studies.

The theoretical results (as well as the experimental points) of the Pb concentrations in bones are displayed in **Figure 3**. Point "0" of the time axis in all figures corresponds to the concentration measured in Control group.

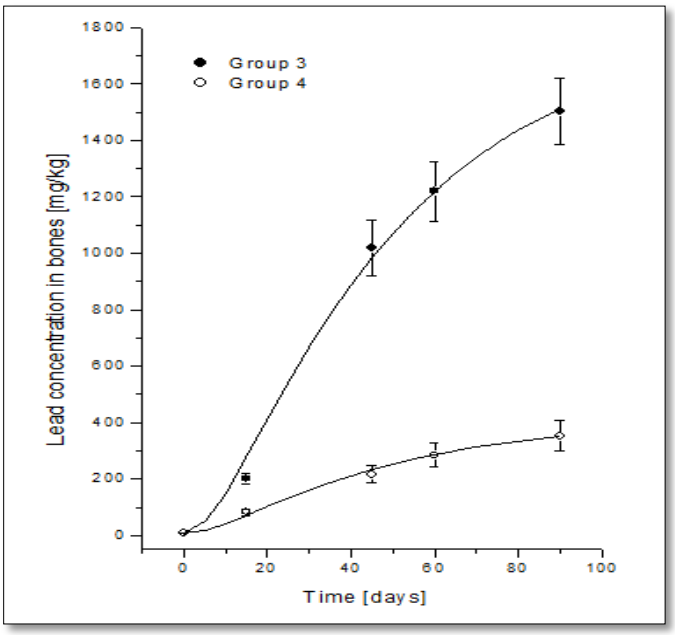

Figure 3. Lead concentrations in bones of ICR mice from Group 3 and Group 4 during the ecotoxicological experiment: Experimental points and model solutions. The time point "0" corresponds to the lead concentration in Control group. The experimental data were recorded at 15th, 45th, 60th and 90th days of the treatment.

The background Pb level in carcass, liver, kidney, bones and feces of the control mice were 0.22 ± 0.06, 0.5 ± 0.07, 0.44 ± 0.08, 9.3 ± 1.3 and 23.6 ± 6.7mg/kg, respectively. On day 90 the Pb concentrations in the mice from 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 mice from Control group. Regarding Group 4, the Pb concentrations were 237-fold, 17-fold, 125-fold, 357-fold and 51-fold higher compared to Control group (Group 1). Significant differences ($p>0.001$) were established in carcass, kidney, bone and feces between groups 3 and 4 after day 15. The reduction in Pb levels in the exposed and supplemented mice compared to exposed non-supplemented ones was as follows: 84%, 89%, 91%, 77% and 88% for carcass, liver, kidney, bones and feces, respectively.

When denote $(Pb_{90}/Pb_{15})_{Group\ 3}=R_3$ and $(Pb_{90}/Pb_{15})_{Group\ 4}=R_4$, the following relations could be constructed:

$$(R_3/R_4)_{Carcass}=2.7;\ (R_3/R_4)_{Feces}=2.19 \qquad (15)$$

$$(R_3/R_4)_{Liver}=11.27;\ (R_3/R_4)_{Kidney}=3.77;\ (R_3/R_4)_{Bone}=1.74 \qquad (16)$$

As seen from (15) and (16), the most value has the relation R_3/R_4 calculated about the liver. It is an expected result: The liver is "the purification plant" of the organism and there the highest toxicant concentration should be registered.

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

Group 3 $Pb_{Bone}/Pb_{Liver}=19$; $Pb_{Bone}/Pb_{Kidney}=2.5$; $Pb_{Kidney}/Pb_{Liver}=7.6$ (17)

Group 4: $Pb_{Bone}/Pb_{Liver}=40$; $Pb_{Bone}/Pb_{Kidney}=6.4$; $Pb_{Kidney}/Pb_{Liver}=6.3$ (18)

The most ratio is this between bone and liver (Bone/Liver ratio) in Group 4. This fact confirms the significant role of the clinoptilolite to avert the reaching of high lead

concentrations in the soft tissues. The relatively low value of Bone/Kidney ratio in Group 3 indicates that the kidneys take much larger part of the Pb load compared to the liver.

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

Bone $Pb_{Group\ 3}/Pb_{Group\ 4}=4.3$ (19)

Liver $Pb_{Group\ 3}/Pb_{Group\ 4}=9$ (20)

Kidney $Pb_{Group\ 3}/Pb_{Group\ 4}=11$ (21)

The ratios for the liver and especially for the kidney are rather high compared to the bone-ratio. This fact once more points out the power of the clinoptilolite sorbent as a means for detoxification of the soft tissues in conditions of lead bioaccumulation. Also, the relatively low bone-ratio is in agreement with the fact that Pb is a bone seeking element.

BLOOD PARAMETERS (ERYTHROCYTE MORPHOLOGY AND ERYTHROPOIESIS)

Lead influence on erythropoietic bone marrow cells causes morphological changes: Microcytic and hypochromic erythrocytes appear. More than 90% of Pb in blood accumulates in erythrocytes and there are at least two major compartments for Pb in red blood cell: one associated with the membrane and the other associated with the hemoglobin. The increased mechanical fragility of cell membrane shortens the erythrocyte lifespan. Depression of the chemosynthesis by Pb is due to the inhibition of SH-containing enzymes controlling it in bone marrow [42]. In addition, the decrease of the hemoglobin level is due to the decrease of the number of dividing erythroblasts and consequently newly-formed erythrocytes. All these features indicate a lead-induced anemia. In our experiment, a drop in the proliferative activity of the bone marrow stem cells and well expressed anemia were clearly observed in the Pb-exposed mice from Group 3.

The hematological analysis was carried out on the same groups of animals using standard clinical methods. Peripheral blood samples were collected between 9 and 11 AM from the orbital sinus [10]. The percentages of various forms of cells were determined using Giemsa stains. About 150-200 cells were counted in each stain.

As known, the hemoglobin level and erythrocyte number are most often investigated. Our attention here was focused mainly on the changes of the erythrocyte morphology because of its usefulness as a clear indicator for Pb intoxication.

The data of the quantitative erythrocyte morphological analysis in our experiment are presented in **Figure 4**. 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 ($p<0.001$). The normal and pathological erythrocytes decreased and increased, respectively, in Group 4, but the normal red blood cells in Group 4 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.

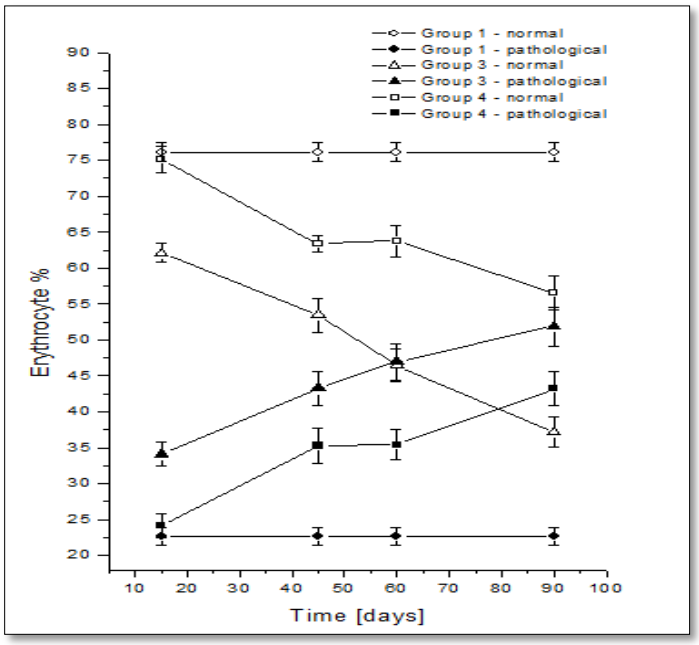

Figure 4. Percentage of the normal erythrocytes and erythrocytes with pathological changes in ICR mice during the 90-day ecotoxicological experiment.

Between groups 3 and 4 and Control group (Group 1), on day 90, the following ratios were established regarding the normal and pathological erythrocytes, respectively:

$$N_1/N_3=2, \quad N_1/N_4=1.35 \qquad (22)$$

$$P_1/P_3=0.43, \quad P_1/P_4=0.53 \qquad (23)$$

Between groups 3 and 4 the following relations were determined on days 15 and 90:

Normal Er: $N_4/N_3=1.2$ (15), $N_4/N_3=1.5$ (90) (24)

Pathological Er: $P_4/P_3=0.7$ (15), $P_4/P_3=0.83$ (90) (25)

The proportion between the pathological erythrocytes in Group 4 and Group 3 remained up to the end of the experiment less than unity, i.e., the quantity of the pathological erythrocytes in Group 4 was less than the same quantity in Group 3.

These results one more time indicate the importance of the clinoptilolite treatment.

A significant drop in the erythropoiesis rate was recorded in the mice from Group 3. Statistically significant differences ($p<0.05$) were observed on days 15 and 90 between groups 3 and 4. As expected, the percentages proliferating erythrocytes in Pb-exposed and supplemented mice (Group 4) were higher than those in Pb-exposed mice without clinoptilolite supplement (Group 3).

The differences between groups 3 and 4 and Control group, especially on day 90, were quite significant ($p<0.001$). Comparing PE in the control mice to PE in the mice from groups 3 and 4 on day 90 the following interrelation was obtained:

$$PE_1/PE_3=10, \qquad PE_1/PE_4=3.2 \qquad (26)$$

The first relation of (26) indicates a 10-fold drop in the number of dividing erythroblasts in Group 3 compared with Control group. The supplementation with KLS-10-MA reduced the respective ratio in Group 4 almost by three times.

CHROMOSOME ABERRATIONS FREQUENCY

Chromosome aberrations are a sensitive endpoint for detecting genotoxic effects induced by various harmful agents, including heavy metals and toxic chemicals [43]. Chromosome aberration frequency (CAF) could be used as an effective biomarker (indicator) of genetic injuries.

The cytogenetical analysis of the samples was performed 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 eight 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°C 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 [14].

The results are presented in **Figure 5**. It is remarkable that no statistically significant differences were observed between CAF in Group 4 (intoxicated and supplemented animals) and CAF in Group 2 (healthy and supplemented animals) except on day 60 ($p<0.05$). Significant differences ($p<0.001$) were recorded between Group 3 and other groups at each time points. The maximum CAF in groups 3 and 4 were observed on day 60, when the following correlations take place:

$$CAF_3/CAF_1=4.56; \; CAF_4/CAF_1=2; \; CAF_3/CAF_4=2.3 \qquad (27)$$

These results underline once again the essential effect of the clinoptilolite sorbent in the reducing of the lead intoxication.

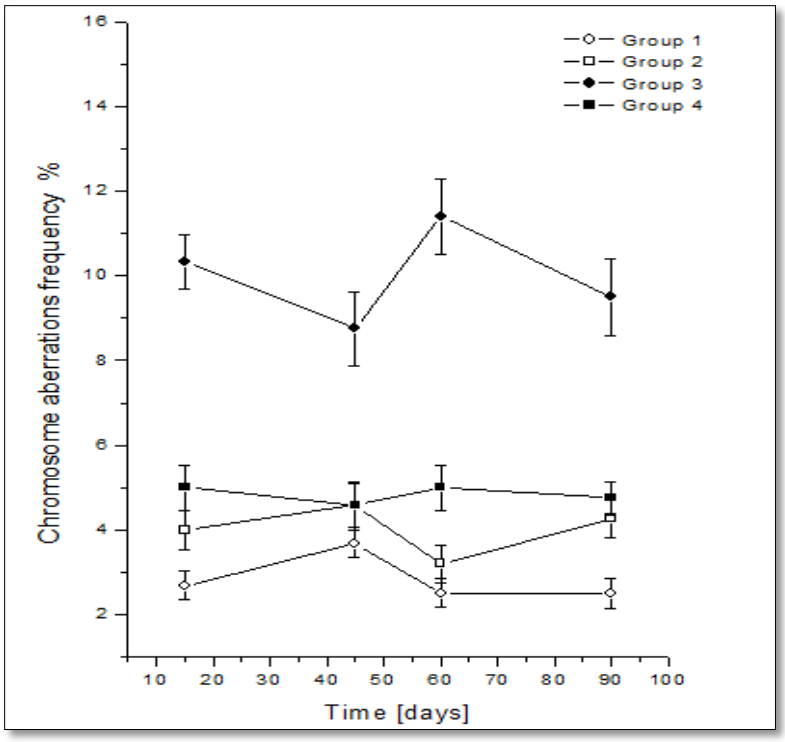

Figure 5. Frequency of chromosomal aberrations in the control and experimental ICR mice.

The distribution of the different type chromosome aberrations is displayed in **Figure 6**. Chromosome breaks and Robertsonian translocations or c/c fusions were the predominant harmful effects of the mouse's genetic apparatus. They had maximal values in Group 3, on day 15: 4.7% and 5%, respectively. This fact indicates that the chromosome injuries appear and increase rapidly from the beginning of the lead treatment and demonstrates the great sensitivity of the genetic apparatus to toxic substances. Fragments were found below 1.5%. Only in Group 3, on day 60, they showed its highest value (3%).

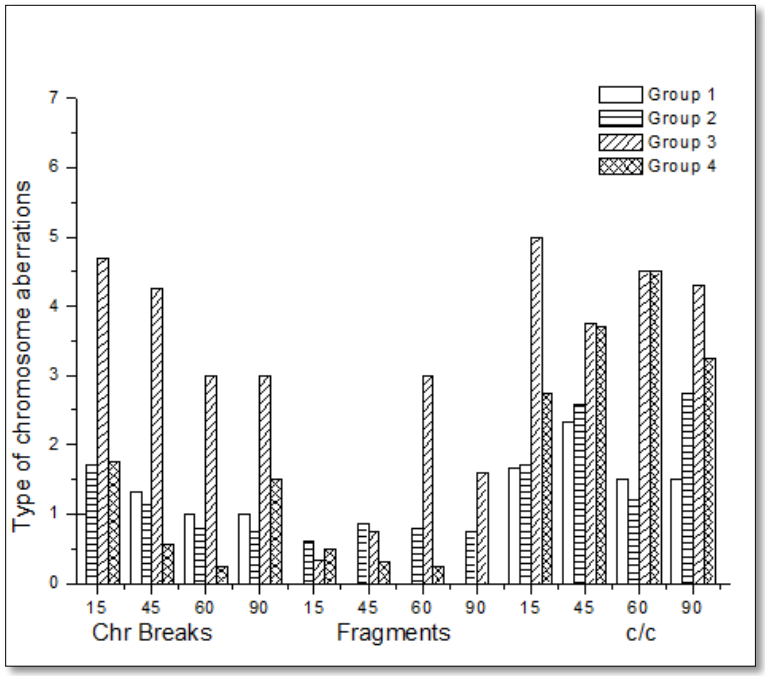

Figure 6. Distribution (%) of the different type chromosomal aberrations in ICR mice during the experiment.

These results allow make the following conclusions:

1. The genetic apparatus of the mouse's organism is very sensitive and fast reacting. (This is a well-known fact. Because of that the rodents are the preferable experimental animals).
2. The genetic apparatus of the mouse's organism reacts to toxic substances mainly via chromosome breaks and Robertsonian translocations.
3. The treatment with the clinoptilolite sorbent significantly reduces the chromosome breaks and fragments and to a lesser degree the Robertsonian translocations.

KINETICS OF THE LEAD BIOACCUMULATION

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

$$z(t) = z_0 + Aa_1a^3\left(\frac{1}{b_1b_2} - \frac{1}{b_1(b_2-b_1)}e^{-b_1t} + \frac{1}{b_2(b_2-b_1)}e^{-b_2t}\right) \quad (8)$$

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

The solution $z(t)$ represents the process of Pb bioaccumulation in the bones of the mice. The graphical time course of bone Pb is presented in **Figure 4**. 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 η=15% it is 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_{Group3}=58.6 mg. Coefficient η (0<η< 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 $A_{Group3}=x(t_0)$=1953 mg/kg. The parameters were fitted by minimization of 2 by the use of an iterative Gauss-Newton procedure [45,46]. 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: $A_{Group4}=x(t_0)$=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}.

The model shows that the rate constants of Pb excretion by the feces a_2 in Group 4 are twofold higher than that in Group 3: $a_{2\ Group4}=2a_{2\ Group\ 3}$. This result corresponds to the real situation, because as a ballast matter, the clinoptilolite sorbent accelerates the intestine passage.

The mathematical model of Pb kinetics is in good agreement with the experimental data for Pb concentration in bones. This model allows determining the coefficient of Pb gastrointestinal absorption (absorption coefficient) in the experimental animals from Group 4, using the formula:

$$\eta = \frac{A_{Group_4}P}{1000BT} \quad (29)$$

where $A_{Group\ 4}=x(t_0)_{Group\ 4}$ is the concentration of lead absorbed in the digestive tract and entering the blood extrapolating over the experiment (for the mice of Group 4), 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 value η=3.53% obtained in clinoptilolite supplemented mice (Group 4) is 4.25 fold lower compared with η=15% in non-supplemented animals. A reduction of 76% occurred! In fact, this is a significant result: KLS-10-MA diminished the Pb absorption in gastrointestinal tract of mammals' organism more than four times! This result allows prognosticate an excellent perspective for the application of the clinoptilolite sorbent in mammals.

KINETICS OF THE MITOTIC INDEX

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 was statistically evaluated for their significance by analysis of variance using student t test.

A trend of continuous decrease of the mitotic index during the experiment was established in the exposed-non-supplemented mice. Contrariwise, in the exposed-supplemented mice, the level of the mitotic index decreased up to day 45 but then steadily increased ($p<0.001$) **(Figure 7)**.

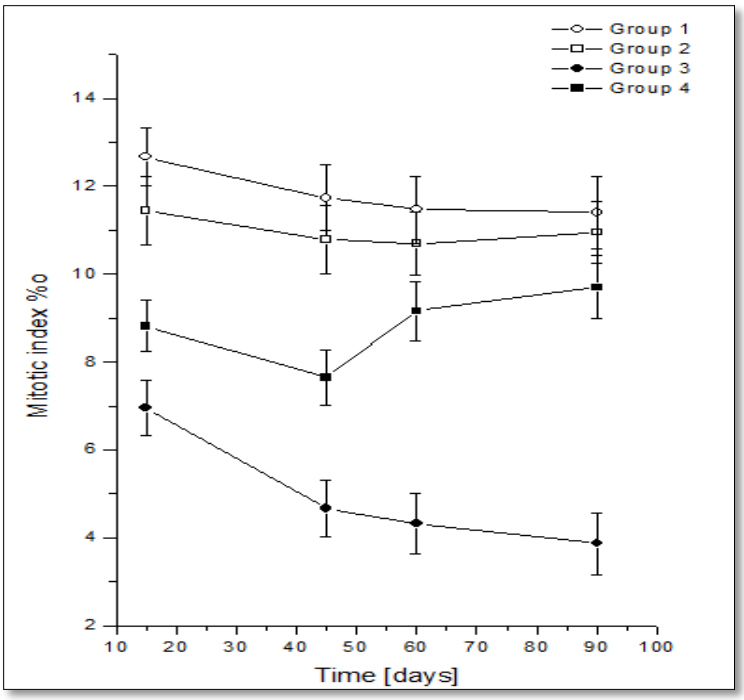

Figure 7. Mitotic index determined in bone marrow cells of ICR mice during the experiment. The point "0" corresponds to the mitotic index in the mice from control group.

The following comparisons were made and respective ratios calculated regarding the mitotic indices (M) (the time points are given in brackets):

M_1/M_3=1.8 (15) M_1/M_3=2.5 (45) M_1/M_3=2.7 (60) M_1/M_3=2.9 (90) (30)

M_1/M_4=1.4 (15) M_1/M_4=1.5 (45) M_1/M_4=1.3 (60) M_1/M_4=1.2 (90) (31)

M_4/M_3=1.3 (15) M_4/M_3=1.6 (45) M_4/M_3=2.1 (60) M_4/M_3=2.5 (90) (32)

The mitotic indices in the mice from Group 4 in all points of the observation were close to those in Control group. The relations M_4/M_3 steadily increased during the experiment and this indicates the determinant role of the clinoptilolite sorbent in the adsorption of the toxicant and therefore in the improvement of the genetical status in the mice from Group 4.

In the 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 (Group 3):

$$\frac{dm}{dt} = -a(t)m \qquad (33)$$

Under initial condition:

t_0=0, $m\ (t_0)=m_0$ (34)

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]=[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 $a\ (t)$ should decrease with the time. For $a\ (t)$, under initial condition: t_0=0, $a\ (t_0)=a_0$, we found:

$$a(t) = a_0 e^{-rt} \qquad (35)$$

Taking into account of (35), the differential equation (28) could be written in the form:

$$\frac{dm}{dt} = -a_0 e^{-rt} m \qquad (38)$$

The following differential equation is adequate to describe MI behavior in the case of clinoptilolite supplementation (Group 4):

$$\frac{dm}{dt} = -a_0 e^{-rt} m + k(t) \qquad (39)$$

Under initial condition:

$t_0 = 0$, $m(t_0)=m_0$ (40)

The parameter k ([k]=[% day^{-1}]) could be named recovery rate and it could be considered as an integral characteristics of the complex of recovery processes developing in 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.

Finally, for $m(t)$ one can write:

$$\frac{dm}{dt} = -a_0 e^{-rt} m + k_0 e^{-ct} + \frac{g}{c}(1 - e^{-ct}) \quad (41)$$

The solution **(Figure 7)** of the equation (39) for the unsupplemented mice (Group 3) under initial condition (40) is:

$$m(t) = m_0 e^{-\frac{a_0}{r}(1-e^{-rt})} \quad (45)$$

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

The equation (44), for the time course of MI in the mice from Group 4, cannot be solved analytically and a numerical solution was obtained. It is presented in **Figure 8**.

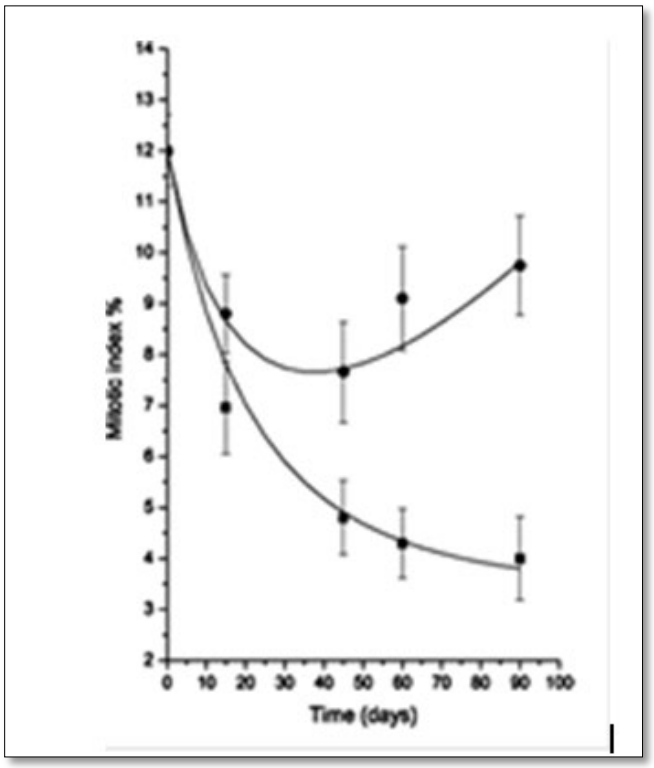

Figure 8. Mitotic index $m(t)$ in the bone marrow cells of ICR mice from Group 3 (■) and Group 4 (●) during the experiment: Model solutions and experimental points. The point "0" corresponds to the mitotic index in the mice from Control group.

In the observed mitotic indices no statistically significant differences were established ($p>0.1$) between Control group and Group 2. Significant differences ($p<0.05$-$p<0.001$) were found between Group 3 and Group 4, between Control group and Group 4 ($p<0.002$) and especially between Control group and Group 3 ($p<0.001$). On day 90, MI in Group 4 was almost 2.5-fold higher compared to that in Group 3 **(Figures 2 and 7)**. On the same day MI in Control group was almost 3-fold higher than that in Group 3 and only 1.2-fold higher than that in Group 4. A continuous decrease of MI during the experiment was established in the mice from Group 3. Contrariwise, in the exposed and supplemented mice from Group 4, MI decreased up to day 45 but then steadily increased.

BODY WEIGHT

The body weight is an important biological marker for the estimation of the vital status of the living organisms in different injuries, including metal intoxication. Each intoxication causes damages on the organs and tissues in the animal organism and reflects particularly on the growth and biomass.

Many researches have proved that the dietary inclusion of zeolites, particularly clinoptilolites, as a feed additive in animal's nutrition improves body mass, average daily gain and/or feed conversion ratios in pigs, ruminants, sheeps, poultry, broilers [31,47,48]. Apart from the positive effects on animal's performance, dietary supplementation of

zeolites appears to represent an efficacious, complementary, supportive strategy in the prevention of certain diseases and the improvement of animal's health status.

The changes with time of mean body weight of the animals are presented in **Figure 2**.

CONCLUSION

The inclusion of the modified clinoptilolite sorbent KLS-10-MA (prepared, based of natural Bulgarian zeolite) as a food supplement significantly decreased Pb concentrations in the body and organs of the experimental animals, loaded with lead. This is due to the fact that the clinoptilolite sorbent, as a reliable absorbent, trapped in the stomach a great part of lead and thus limited the Pb entering the blood. In the sorbent-supplemented animals the bioaccumulation coefficients is $\eta=Pb_{90}/Pb_{15}$ =3.53% [49-55]. (Pb_{15} and Pb_{90} are the respective lead concentrations on days 15 and 90). (This value is about 4 times lower compared to the known coefficient for Pb absorption in non-treated animals: 15%). In the supplemented mice the lead concentrations in carcass, liver, kidneys, bones and feces decreased by 84%, 89%, 91%, 77% and 88% respectively, compared to those in the unsupplemented ones [56-60].

The structure of the chromosomes and red blood cells as well as the mitotic index and erythropoiesis, also the body mass, were significantly improved in the group of the intoxicated mice, supplemented with KLS-10-MA. These mice appeared active and healthy [60-63].

The mathematical model for the lead bioaccumulation 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 [64].

The mathematical model for the mitotic index allowed made an exact quantitative evaluation on the positive effect of the clinoptilolite treatment. The theoretical results could also help in order to a more precise determination of the doses of the clinoptilolite supplement. The model confirms the authors' hypothesis that the sensitivity of the bone marrow cells to the toxicant and the recovery rate of the genetic apparatus remain not constant with the time. Really, the sensitivity decreased and the recovery rate increased with the time due to the stimulated defense reaction of the organism. The model could predict the state of cell proliferation in each time point of the observation and allows a quantitative evaluation of the positive effect of the treatment. Such models could help in the toxicological investigations [65-67].

This study demonstrates a great benefit of the clinoptilolite as a food additive. We have every reason to conclude that the used clinoptilolite sorbent KLS-10-MA is quite effective in gastric juice medium and could be excellent food additive for a wide application in mammals, under conditions of Pb intoxication and perhaps in a wide range of influences of toxic substances. Besides, the clinoptilolite sorbent exerts a significantly favorable effect on the mammal organisms. In fact zeolites reveal "magical" features!

Clinoptilolite substances could be valuable in agriculture and livestock as well as in human medicine in cases of chronic lead intoxication. The present work gives ground to create a new effective drug based on the clinoptilolite sorbent for supplementation of animals in regions that are industrially polluted with heavy metals and particularly with lead, in order to protect the animals' health and the quality of the environment.

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