Zeolites versus Lead Toxicity

Beltcheva M1*, Metcheva R1, Topashka-Ancheva M1, Popov N3, Teodorova S2, Heredia-Rojas JA4, Rodriguez-de la Fuente AO4 and Rodriguez-Flores LE5

1Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1, Tzar Osvoboditel Blvd., 1000 Sofia, Bulgaria
2Institute for Nuclear Research and Nuclear Energy, Bulgarian Academy of Sciences, 72, Tzarigradsko shaussee Blvd., 1784 Sofia, Bulgaria
3Mineralagro LTD, 53, Cheni vrh Blvd., 1407 Sofia, Bulgaria
4Departamento de Ciencias Exactas y Desarrollo Humano, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Av. Pedro de Alba y Manuel L. Barrágán s/n Cd. Universitaria, C.P. 66451, San Nicolás de los Garza, Nuevo León, México
5Departamento de Patología, Facultad de Medicina, Universidad Autónoma de Nuevo León, Av. Francisco I. Madero s/n y Av Gonzalitos, Col. Mitrás Centro. C.P. 66460, Monterrey, Nuevo León, México.

Abstract

The workers in metal industry as well as the people inhabiting industrially polluted regions are Health hazard groups regarding the development of chronic heavy metal intoxication. Different means could be used to correct, at least partly, the consequences of such intoxication. However, the best substances for treatment are those that can prevent the metals entering in the blood. In case, metals enter in the organism through the digestive tract, zeolites are the most suitable means for trapping of metal ions. The substantial role of the clinoptilolite as a factor essentially reducing Pb bioaccumulation is considered in an experiment with small mammals chronically exposed to lead.

As a feed additive, clinoptilolites have been used so far in poultry and livestock to positively influence feces consistency, reduce diarrhea, bound mycotoxins and aflatoxins, and allow better performance of intestinal microflora. The present work is the first study of the effect of clinoptilolite, used as a food supplement, in conditions of Pb intoxication.

Modified clinoptilolite KLS-10-MA was prepared and applied as food-additive in laboratory inbred ICR line mice, chosen as experimental animals. In the experiment the degree of the positive effect of this sorbent in the reduction of Pb bioaccumulation was explored. Evidences that clinoptilolite is practically non-toxic substance were presented. A mathematical model of Pb bioaccumulation in exposed and exposed-supplemented animals was proposed.

Such investigations are important for human and veterinary medicine, pharmacy and for the explanation of some biological and chemical problems. The authors hope that the obtained results could help in further efforts to create drugs based on clinoptilolite sorbents. An application of such drugs could be of great importance for human and animals in regions that are industrially polluted with heavy metals, and particularly with Pb, in order to protect the organisms as well as the quality of the environment.

Keywords: Bioaccumulation; Intoxication; Clinoptilolite sorbents

Introduction

Zeolites-natural and modified because of their specific structure, are excellent adsorber and thus can diminish the harmful effect of heavy metals. Clinoptilolites, due to its structural stability under high temperatures and acidity, are the most widely used zeolites in animal studies. The important research data are presented, indicating the positive influence of the in-feed inclusion of clinoptilolite on animal health.

Ecotoxicological experiment with laboratory inbred ICR line mice was carried out, covering 90 days. The mice were chronically exposed to lead (Pb) in the form of aqueous solution of Pb(NO3)2, diluted in the drinking water, and treated with modified clinoptilolite sorbent KLS-10-MA. They were allocated into four groups: group 1, (control): animals fed with conventional food + KLS-10-MA and water; group 2: animals fed with conventional food + clinosorbent KLS-10-MA and water; group 3: animals fed with conventional food + clinosorbent KLS-10-MA and water + Pb(NO3)2; and group 4: animals fed with conventional food + Pb(NO3)2. Group 2 was a second control in order to prove eventual toxicity of the sorbent and influence of clinoptilolite on growth performance. To assess the detoxification effect of the clinoptilolite sorbent, the following bioindicator characteristics were chosen: Pb concentrations in carcass, organs, tissues, and feces, chromosomal aberrations frequency, mitotic index, erythrocyte morphology, erythropoiesis, and body weight gain of the mice.

The modified clinoptilolite KLS-10-MA (water modification) was prepared on the base of natural Bulgarian clinoptilolite from the deposit in East Rhodops, South Bulgaria. As a powder, it was mechanically mixed at 12.5% concentration with the conventional forage for small rodents and was administrated as feed supplement for the mice from groups 2 and 4. A significant reduction of Pb bioaccumulation in the exposed and supplemented mice (group 4) was observed: the Pb levels in the mice from group 4 were 84%, 89%, 91%, 77%, and 88% lower for carcass, liver, kidney, bones, and feces, respectively, compared to the Pb levels in the mice from group 3. Essential improvement of the cytogenetical, hematological, and physiological parameters was noted. On day 90 the following relations were calculated: Pb-exposed and clinoptilolite-supplemented mice exhibited 2.3-fold lower chromosome aberrations frequency, 2.5-fold higher mitotic index, 1.5-
fold higher percentage normal erythrocytes, and 1.3-fold higher body weight compared to Pb-exposed and unsupplemented animals.

The obtained results support the suggestion of other authors that clinoptilolite is quite effective in gastric juice medium and could be suitable for wide application in mammals under conditions of Pb poisoning.

No data were found about applications of zeolites as feed additive in animals exposed to heavy metals. The proposed exploratory research about detoxification capacity of the clinoptilolite is the first step in this direction.

 Adequate mathematical model was constructed to explain the main regularities in Pb bioaccumulation in the bones. Based on the experimental data the kinetic parameters of the Pb accumulation and lead excretion in mouse's organism were fitted. A value of gastrointestinal Pb resorption coefficient in KLS-10-MA-supplemented mice $\eta = 3.53\%$ was calculated versus normal value $\eta = 15\%$.

The results of the presented study encourage the further efforts for the finding of reliable drug based on the tested clinoptilolite substance in cases of chronic lead intoxication in animals and human.

Heavy metals intoxication is a serious social problem. It directly affects humans or animals health and due to the withdrawal of ill persons from the active work in companies for processing of nonferrous metals also the production affects. Heavy metals in the form of aerosols and dust, especially in industrial areas fall into the water, soil, air, and plants. It creates unfavorable environmental situation.

In order to minimize risks to life, health and working capacity of the population, it is necessary to find methods of reducing harmful effect of heavy metals. Such agents may be sought in three major directions:

1) Decreasing the harmful emissions from the factories by using special purification filters.

2) Use of appropriate materials with specific properties and strongly expressed absorptive capacity to cleanse indoor air pollution in industrial companies, wastewater treatment, soil and fertilizers.

3) Use of suitable substances with strongly expressed absorptive capacity for internal application in humans and animals in order to neutralize heavy metals that fell within the organism to minimizing their resorption from the gastrointestinal tract.

One of the best means suitable in cases 2) and 3) are the zeolites. Series of ecotoxicological experiments and careful studies have confirmed their high efficacy as adsorbers of waste products [1-3]. Clinoptilolites are often utilized to draw heavy metals from solutions [4-6] and to purify deleterious emissions [7]. Diets manipulated by zeolites have the potentiality to reduce both the excess of N and P in swine manure, and to minimize the negative effects of odor and other gaseous emissions such as $\text{NH}_3$ and $\text{H}_2\text{S}$ from the swine waste [8,9]. As a feed additive, clinoptilolites have been used so far in poultry and livestock in order to positively influence feces consistency, reduce diarrhea, bound mycotoxins and aflatoxins, allow better performance of intestinal microflora [10]. No data were found about applications of zeolites as feed additive in animals exposed to heavy metals. This exploratory research was the first step in this direction. Clinoptilolite was used as food supplement in laboratory mice, in conditions of chronic Pb-exposure. An effect of essentially diminished Pb toxicity in experimental animals was established.

Lead is one of the most popular environmental pollutants resulting from anthropogenic activities. It predominates as a toxic agent in industry. Lead along with cadmium and mercury is the most toxic and highly deleterious heavy metal. It causes alterations in growth and behavior, renal function deficits, hypertension, osteoporosis, lead-induced anemia, etc. in human and animal organisms. [11-13]. Lead is also a significant genotoxic agent [14-16]. Therefore, it is of great importance to take pains for possible reduction of lead toxicity.

Clinoptilolites are minerals with specific crystalline structure, which makes them a perfect “heavy metals trap” [17-19]. Besides, they are the most abundant natural zeolites, occurring in volcanic and sedimentary rocks [1,7] and this fact facilitates their use [20] established that clinoptilolite retains its structural stability under high temperatures and acidity. According to there experiments clinoptilolite exhibited the highest capability in adsorbing 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 authors suggest that a significant part of lead (Pb$^{2+}$) intake is possible to be neutralized in the stomach by a clinoptilolite sorbent. Thus, clinoptilolite, which reveal a unique selective adsorption, could be considered as a reliable means to diminish Pb intoxication.

When clinoptilolite is applied, as a food supplement, the process of ions exchange occurs in the gastrointestinal tract and thus the resorption of toxic metals through the intestinal mucosa could be prevented in a great extent. The present information is targeted to explore the degree of the positive effect of a modified clinoptilolite sorbent KLS-10-MA (water modification). The sorbent was administrated as feed-additive in laboratory inbred ICR line mice in condition of chronic poisoning by Pb. Clinoptilolite is practically a non-toxic substance [21]. This statement was confirmed also by present results.

Laboratory inbred ICR line mice, were chosen as experimental animals. Small mammals, and especially rodents, are preferred as bioindicators in ecotoxicological experiments due to their basic position in food chain, fast reaching of maturity, high total metabolism and specific biological reactions (substantial increase of chromosome aberrations frequency, changes of hematological indices etc.).

In the present study bioaccumulation of Pb in carcass, organs and tissues, as well as chromosomal aberrations and mitotic index, erythrocyte morphology and proliferation, and body weight gain of the mice were chosen as suitable bioindicator characteristics to assess the detoxification effect of the modified clinoptilolite sorbent KLS-10-MA administrated as a feed supplement in the mice chronically exposed to Pb.

The mathematical model of Pb bioaccumulation in bones, in exposed-unsupplemented and exposed-supplemented mice, allowed determining the coefficient $\eta$ of absorption of Pb by gastrointestinal mucosa in the supplemented mice and some kinetic parameters of the dynamics of Pb bioaccumulation both in unsupplemented and supplemented animals. The quantitative investigations of the impact of KLS-10-MA on cell and physiological parameters during a chronic Pb exposure of small mammals could help in further explorations to use this sorbent against chronic metal poisoning.

Other aspect is also in the scope. Many authors report the positive role of zeolites in livestock breeding [22-26]. 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. These were
healthy animals non-exposed to Pb and fed with conventional forage mixed with 12.5% KLS-10-MA.

Zeolite structure and the use of zeolites as excellent adsorber of waste products and heavy metals

Zeolites are an excellent “trapper” of waste products and heavy metals because of their chemical composition and specific lattice structure. These minerals are crystalline, hydrated aluminosilicates of alkali and alkaline earth earth cations (Na, K and/or Ca cations). Zeolites have an infinite, open structure [2]. They consist of threedimensional frameworks of SiO₄⁻ and AlO₄⁻ tetrahedra linked through the shared oxygen atoms. Both natural and synthetic zeolites are porous materials, able to adsorb molecules of appropriate cross-sectional diameter and to exchange their constituent cations without major change of their structure. Thus, zeolites appear to possess two important properties: adsorption property and ion-exchange property [17]. The exploitation of these properties underlies the use of zeolites in a wide range of industrial and agricultural applications and particularly in animal nutrition since mid-1960s [2].

The most important of zeolites are: analcime, chabazite, clinoptilolite, erionite, faujasite, ferrierite, heulandite, laumontite, mordenite, phillipsite. With large size deposits and wide geographic distribution, clinoptilolites are considered as most abundant members of the 48 minerals in the zeolite group [18,27]. Clinoptilolite usually occurs in volcanic and sedimentary rocks [1,7]. Due to its abundance, wide dissemination and ion exchange characteristics, clinoptilolite minerals are among the most often used zeolites.

As zeolite, clinoptilolite is crystalline, hydrated aluminosilicates of alkali and alkaline earth earth cations, consisting of three-dimensional frameworks of SiO₄⁻ and AlO₄⁻ tetrahedra linked through the shared oxygen atoms. The molar Si/Al ratio in clinoptilolites is above 4 [28,29]. Clinoptilolites have a relatively open structure with a total pore volume of approximately 35% [30], and chemical formula (K, Na, Ca, Mg), (AlSi₃O₈)·5H₂O [2]. The structure of clinoptilolite is characterized by large intersecting open channels of 10- and 8-member tetrahedral rings [20]. Such structure ensures the clinoptilolite capacity of to absorb and accumulate heavy metals. Clinoptilolites are often utilized to reduce them from solutions [3,4,6,31].

Why are clinoptilolites a perfect heavy metal-trap? The Si-block is neutral, while the Al-block in crystalline unit 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 are exchanged in solutions with cations of certain metals, such as Pb, Cd, Hg, etc [18,19].

In order to meet increasingly stringent Environmental Quality Criteria (EQC) clinoptilolites are widely applied for heavy metals removing from polluted streams and wastewater [4,19,32-37] and for the elimination of gas pollutants in confinement facilities [7,10]. Besides, this mineral has widespread applications in agriculture [1,2]. According to [38], zeolites also allow better performance of intestinal microflora.

The effects of clinoptilolites in animals appear to be related to their high cation-exchange capacity, which affects tissue uptake and utilization of NH₄⁺, Pb²⁺, Cd²⁺, Cu²⁺, Cs⁺, and other cations [6,19]. Clinoptilolites have shown antioxidant and anticancer effects [39-41]. They support the immune activity [20,42,43] note that clinoptilolite with low toxicity and high adsorption capacity toward Pb²⁺ in a strong acid solution has a potentiality for application in the life sciences. Clinoptilolites appears to be stable in the gastrointestinal tract [44], and as unique selective adsorbers, they could adsorb toxins, heavy metals, and free radicals from the body and excretes [17,45]. It is believed that the high affinity of clinoptilolite to Pb would significantly reduce the amount of dietary Pb available for absorption by the intestinal mucosa [20]. Present results confirmed this suggestion. The lead ions could be “trapped” by clinoptilolites in the stomach because the obtained Pb concentrations in exposed and clinoptilolite-supplemented animals were much lower compared with those in exposed unsupplemented ones. The sorbent KLS-10-MA (water modification) appeared as high reliable means for detoxification of animal organisms chronically poisoned by lead. Preparation of modified natural clinoptilolite for the experiment

In Bulgaria, clinoptilolite are of most widespread distribution in the north-eastern Rhodopa Mountain. For the preparation of the modified clinoptilolite KLS-10-MA (water modification) natural clinoptilolite from the deposit in the village Golobradovo was used. Golobradovo is situated in the region of East Rhodops, South Bulgaria (Figure 1). The chemical compositions of this clinoptilolite and the modified clinoptilolite KLS-10-MA are presented in Table 1. The values of exchangeable alkaline and alkaline earth earth cations are displayed in Table 2.

The modified clinoptilolite was prepared through a treatment of natural clinoptilolite (zeolite containing 82% clinoptilolite). The natural clinoptilolite was heat-treated at 240-250°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 [46].

Clinoptilolites of high quality occur in Bulgaria [47-49]. Table 1 shows that the molar ratio Si/Al was 5.97 in natural clinoptilolite and 6.22 in modified KLS-10-MA based on this clinoptilolite. A rather...
The sorbent KLS-10-MA used in the present experiment is a Na-enriched one [20] established that the structure variations of Na-clinoptilolite in the acidic solution for 24 h would have a minor influence [20]. The cation exchange capacity of KLS-10-MA was almost 1.4-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. [20,49-51]. Thus, the modification KLS-10-MA could be considered as a successful sorbent for detoxification purposes.

**Experimental Setting**

**Animals and treatment**

The eco-toxicological experiment was conducted according to approved protocols and in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes and the current Bulgarian laws and regulations.

Experimental 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 aclimatized for a 7 days period before starting the experiment. A standard temperature of between 19°C and 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 water, food and bedding material were daily inspected and changed when necessary. The animals were neither medicated nor vaccinated.

The experiment covered 90 days. Only males, about 6–8 weeks of age, laboratory mice, inbred ICR strain, were used. The exposure to Pb was performed as the mice were treated with 0.05 N solution of lead nitrate diluted 1:10 in the drinking water during all the investigating period. The clinosorbent KLS-10-MA in form of powder was mechanically mixed to a 12.5% concentration with conventional granulated forage for small rodents.

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 + KLS-10-MA and water + Pb(NO\(_3\))\(_2\).

Two variants of the feed mixture were prepared: (1) standard (conventional food) and 2) standard food treated with 12.5% sorbent KLS-10-MA. The elements’ composition in the variants 1 and 2 are presented in Table 3.

The concentrations of Pb in the whole body, liver, kidney, bones, and feces of the control and experimental animals, as well as cytogenetical and hematological parameters were determined on days 15, 45, 60, and 90 from the beginning of the experiment.

**Elements content analysis**

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-perchloric acid (4:1) [52]. The concentrations of Pb and the element composition of the two feed 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.

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 were processed according to Kolmogorov-Smirnov test for normality in each group. All groups showed normal distributions. Secondly, the data were analyzed by variance of subsequent Tukey high statistical difference test and Dunnet test, for estimating individual differences.

### Cyto genetic and hematological analyses

Percentages of aberrant mitoses, mitotic indices, and blood samples were determined on days 15, 45, 60 and 90 of exposure. At each time point, a subset of eight mice from the four groups was used.

The cytogenetical analysis was performed as described by [53]. 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.

To perform bone marrow chromosomal aberration assays, 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.

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.

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 [54]. The percentages various form of cells were determined using Giemsa stains. About 150–200 cells were counted in each stain.

For the statistical analysis, calculation of the mean ± SD for each group was performed, and the data were evaluated for their significance by analysis of variance using Student t test.

### Mathematical model for lead bioaccumulation in mice’s bone

The problems of bioaccumulation of heavy metals and toxic elements are closely connected to the health and need quantitative consideration. A mathematical model for the kinetics of lead accumulation in the mice’s bone in conditions without treatment and treatment with clinoptilolite sorbent is elaborated in the present study. The model helps calculate some kinetic parameters characterizing the Pb bioaccumulation status and the extent of the sorbent effect.

There are not enough studies that suggest quantitative approach via mathematical modeling of the processes of metals bioaccumulation in living organisms. Physiologically based kinetic models (PBKM) for arsenic, chromium, mercury and lead have been proposed with any degree of completeness [12]. A simple mathematical model for Pb concentration in the body of mice, fed with a contaminated diet, was proposed from [55]. Series of PBKM for bone seeking elements [12,56–60], have developed. Distribution and bioavailability of lead, chromium and uranium have been considered [61]. 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 [62]. It was constructed taking into account that these metals induct metallothionein synthesis. The model well explained the observed peculiarities of Cd bioaccumulation pattern in liver and kidney in conditions of very high exposure to heavy metal mixture.

The present mathematical model is confined to Pb bioaccumulation in mouse’s bones. Three “compartments” of Pb movement are considered: gastrointestinal tract, blood and bones (Figure 2). 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:

\[
\frac{dx}{dt} = -a_1 x + a_2 x \\
\frac{dy}{dt} = a_1 x - a_2 y - a_3 y \\
\frac{dz}{dt} = a_3 y
\]

under initial conditions:

\[
t_0 = 0, x(t_0) = x_0 = A, y(t_0) = 0, z(t_0) = z_0
\]

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; \(t_i\) is the moment when the experiment starts; \(a_1, (\{a_1\} = [day^{-1}])\) and \(a_2, (\{a_2\} = [day^{-1}])\) are the rate constants of Pb accumulation in blood and bones, respectively; \(a_3, (\{a_3\} = [day^{-1}])\) 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.

In Chapter 9 the results from this mathematical model, applied to the dynamics of Pb bioaccumulation in mouse’s bones, are presented, compared with the experimental data, and discussed.

### Lead – toxic effects on animals and humans

The distribution, accumulation and transfer of heavy metals,
and death. Peripheral neuropathy is a classic manifestation of Pb irritability, loss of appetite, and dizziness, progressing to obvious to Pb. Symptoms of Pb encephalopathy begin with lethargy, vomiting, multiple hematological effects as well as genotoxic effects [76]. Lead has also reactive oxygen species resulting in oxidative stress, thereby resulting cardiovascular system etc [71]. Lead produces an excessive amount of and organs, including the central and peripheral nervous system, the estimated [79]. Lead affects a wide range of physiological systems population, associated with high Pb tissues concentration has been health [74,76-78]. A significant ecotoxicological risk for a wild interest as a toxicant and a contaminant. It is widely distributed and [11] has presented a detailed view on the toxicokinetics, toxicity and have published on the distribution, lethal doses, toxicokinetics, and mechanisms [76,77]. The toxic action of heavy metals has also been studied. Mechanisms of lead toxicity have been studied at the cellular and subcellular levels [74,75] evaluated the carcinogenic activity of cadmium [73] have published on the distribution, lethal doses, toxicokinetics, and toxicity mechanism of metals and other inorganic compounds. Also, [11] presented a detailed view on the toxicokinetics, toxicity and pathological effects of the metals.

The toxic action of heavy metals has also been studied. Lead, as ubiquitous environmental pollutant, is of particular interest as a toxicant and a contaminant. It is widely distributed and represents a significant toxicological and ecotoxicological risk factor. Its distribution is closely connected with human activities. Because of that chronic Pb intoxication in workers engaged in the respective activities is a serious health and social problem.

Lead has long been recognized as a potential hazard to human health [74,76-78]. A significant ecotoxicological risk for a wild population, associated with high Pb tissues concentration has been estimated [79]. Lead affects a wide range of physiological systems and organs, including the central and peripheral nervous system, the cardiovascular system etc [71]. Lead produces an excessive amount of reactive oxygen species resulting in oxidative stress, thereby resulting in hypertension [13] and chronic kidney disease [80]. Lead has also multiple hematological effects as well as genotoxic effects [76].

Lead encephalopathy may occur in children with high exposure to Pb. Symptoms of Pb encephalopathy begin with lethargy, vomiting, irritability, loss of appetite, and dizziness, progressing to obvious ataxia, a reduced level of consciousness which may progress to coma and death. Peripheral neuropathy is a classic manifestation of Pb toxicity, particularly the footdrop and wristdrop that characterizes the house painter and other workers with excessive occupational exposure to Pb [11].

Lead may affect blood pressure by altering sensitivity of vascular smooth muscle to vasoactive stimuli via the following mechanism: Pb alters calcium-activated functions of vascular smooth muscle cells including contractility by decreasing Na⁺/K⁺-ATPase activity and stimulation of the Na/Ca pump. Lead acts also indirectly by altering neuroendocrine input to vascular smooth muscle [11].

Lead has a harmful effect on bones. It is known that the total body burden of lead is divided into two pools, which have different rates of turnover. The largest and kinetically slowest pool is the skeleton, with a half-life of more than 20 years, and a much more labile (about 20 days) is the soft tissue pool [11,73]. Lead occurs in bone in much higher concentration than in other organs [60,72,73,81] because it is a strongly bone-seeking element [82,83]. Our data support this concept [62]. Well-defined age dependence was found among total bone lead turnover, bone formation rate, and Pb accumulation in bone [59]. [84] note that hair and epidermal compartments (nails etc.) can also accumulate Pb. Much higher concentrations of lead have been observed in the shells than in the soft tissues of some marine shellfish and crustaceans [85]. Obviously, these facts are connected to the behavior of lead as bone-seeking (or more generally, as calcium-formations-seeking) element. The accumulation of lead in metabolically inactive tissues, mainly in bone, may be considered to a certain extent a protective mechanism for organisms. But O’Flaherty and coauthors showed that lead sequestered in bone can become bioavailable in some conditions, related to physiological states or aging [12,86]. A substantial transplacental transfer of endogenous Pb from maternal bone during pregnancy was observed in primates [86] modeled the process of osteoporotic bone loss in aging as a result from the return of biologically significant amounts of Pb to the blood. In agreement with experimental observations, an increase of blood Pb, previously stored in bone, was modeled after menopause. The model predicts a blood Pb concentration of 17.6 g/dl in a 50-year-old US woman and 18.5 µg/dl in a 60-year-old US woman in the period 1976-1978. Lead adversely influences bone development through disruption of mineralization during growth [82]. The trends for the accumulation of Pb in cortical bone and the release of Pb from bone stores are well predicted by the O’Flaherty model of Pb kinetics and model results are consistent with the hypothesis that a polymorphism in the delta-aminolevulinate dehydratase enzyme modifies the kinetics of Pb in humans [87].

Lead nephropathy is one of the oldest recognized health effects of Pb. Lead nephropathy appears as acute (reversible) or chronic (reversible) [11].

Acute Pb nephropathy is limited to functional and morphologic changes in proximal tubular cells. The functional changes are thought to be related to a Pb effect on mitochondrial respiration and phosphorylation. A characteristic microscopic change is the formation of a lead-protein complex, which appears in renal tubular cells as inclusion bodies. Formation of such a complex was also found to occur in renal tubular cells by [88,89]. Also, the primary renal toxicity of cadmium affects proximal renal tubular function and is manifested by increased Cd in the urine. It was supposed [62] that the increased urinary leak (the polyuric stage of nephropathy [90] is the reason for the essential change of the pattern of the Cd kidney/liver ratio (Cdkidney/ Cdliver). There is a strong relation between the intake of Cd in the diet and the levels of this element in the kidney and liver, both responding by accumulating and storing the cadmium [91]. Usually in human Cd concentration in kidney is greater than in liver. Cdkidney/Cdliver is well
above one (about 5) [11]. The same is valid also for rodents [68,91]. However, an essential decrease, even to inversion (well below one) in this ratio has been observed in the conditions of very high Cd exposure [62,91]. Thus, the ratio Cd_{\text{ubat}}/Cd_{\text{swx}} decreases with the increase of Cd contamination degree.

Lead may produce a chronic interstitial nephropathy, most commonly with blood Pb levels greater than 60 μg/dL [92]. However, depressed glomerular filtration rates have been reported in a group of lead-exposed workers whose blood Pb levels were as low as 40 μg/ dL [92]. The risk of death from renal disease increases with increasing duration of employment [92]. It was reported that of 4519 battery plant workers and 2300 lead production workers there was excess mortality from chronic nephritis [92] have established that Pb nephropathy, characterized functionally by depression of effective renal plasma flow, glomerular filtration rate, and maximum glucose reabsorption rate, is associated with prolonged occupational exposure to Pb. There is a relationship between chronic Pb exposure and gouty nephropathy. Gout patients with renal disease have a greater chelate-provoked Pb excretion then do renal patients without gout. Lead reduces uric acid excretion. Elevated blood uric acid has been demonstrated in rats with chronic Pb nephropathy [93].

Severe Pb-exposure effects on health are typically related also to blood pathology. Reticulocytosis, microcytosis, hypochromy, basophilic erythrocytic granulation and hemoglobin decrease indicate a lead-induced anemia, a well-known symptom of chronic Pb poisoning. More than 90% of Pb in blood is in red blood cells (RBC) and there are at least two major compartments for Pb in RBC: one associated with the membrane and the other with hemoglobin [11]. The anemia is caused by two basic defects: shortened erythrocyte lifespan and impairment of heme synthesis. Shortened lifespan of the RBC is thought to be due to increased mechanical fragility of the cell membrane [11]. The shortened lifespan of RBC results in reticulocyte production as a compensatory reaction. The basophilic stippling occurred in the reticulocytes results from inhibition of the enzyme pyrimidine-5-nucleoside (Py-5-N) [94]. There is an inverse relationship between Py-5-N inhibition and blood Pb concentration. The depression of the heme synthesis is due to the inhibition of SH-containing enzymes by Pb [73]. Besides, iron in the form of apoferritin and ferruginous micelles may accumulate in mitochondria of bone marrow reticulocytes in conditions of Pb poisoning Failure to insert iron into protoporphyrin results in the inhibition of SH-containing enzymes by Pb [73]. Besides, iron in the form of apoferritin and ferruginous micelles may accumulate in mitochondria of bone marrow reticulocytes in conditions of Pb poisoning Failure to insert iron into protoporphyrin results in depressed heme formation [11].

Lead bioaccumulation caused essential reduction of the percentage normal erythrocytes and respectively significant increase of the percentage pathological erythrocytes in the peripheral blood [95]. This finding confirms the study of [94] that has been shown that Pb causes morphological changes in erythropoietic bone marrow cells, leading to production of microcytic and hypochromic erythrocytes. Significant decreases in RBC and mean corpuscular hemoglobin and significant increase in the frequency of micronucleated polychromatic bone marrow erythrocytes have been found in Algerian mice (Mus spretus) exposed to Pb [96].

In addition, lead is a genotoxic factor. It damages the chromosomal structure in mammalian cells [15,16,97-101]. Mutagenicity and carcinogenicity of Pb have been reported by [102] as well as by [103]. At toxic doses, lead acetate and lead nitrate have induced DNA breaks determined by nick translation [104]. Lead essentially reduces the proliferative activity of the bone marrow cells [105]. [99] noted that lead and cadmium cause DNA strand breaks and chromosomal aberrations (CA) only after treatment with high, toxic doses, however, both metals exert pronounced indirect genotoxic effects, which may be due to an interaction with DNA repair processes. The inhibition of DNA repair could play a role in the accumulation and stability of DNA damage, resulting in initiation of carcinogenic process.

Chromosomal aberrations (CA) are changes in normal chromosome structure or number. The structural CA are two main types: breaks and exchanges. The numerical CA are aneuploidy and polyploidy. CA can be formed also spontaneously e.g. from double-strand breaks, generated by cellular events such as topoisomerase action, DNA replication, transposable elements, and fragile sites, and in excision repair of oxidative DNA damage [106]. The comparison of chromosome aberration frequency (CAF) in spontaneous and agent-induced cases allows assessing the extent of harm of the respective agent.

The numerous and various adverse health effects caused by exposure to lead have motivated an earnest quest to find an effective agent that could, albeit partially, prevent the harmful influence of lead on humans and animals. Zeolites have proven to be such suitable agent.

### Toxicity test for clinoptilolite

To calculate the mean Lethal Dose (LD_{50}) of any toxicant, the Karber-method was used as the most punctual method, allowing determination of reliability interval. This method requires an equal number of animals in each group. LD_{50} is calculated by the formula:

\[ \text{LD}_{50} = \text{LD}_{100} \times \left( \frac{z}{d} \right) \ \text{m} \]

where \( n \) = const is the number of animals in a given group; \( z \) (\( z_1, z_2 \), etc.) is the number of dead animals from two neighboring groups divided in two; \( d \) (\( d_1, d_2 \), etc.) is the dose in mg (mL) between two neighboring doses:

\[ z = z_1, d_1 + z_2, d_1 + z_3, d_1 + z_4, d_1 + z_5, d_1 \ldots \]

The test was conducted in order to establish the Lethal Dose (LD_{50}) of the clinoptilolite sorbent administrated to the mice. The data of the investigation on chromosome aberrations, some blood parameters and body weight gain suggested that the used clinoptilolite sorbent was practically non-toxic. A lack of toxic effects of clinoptilolite has been also demonstrated by other authors: [2,45,107-109].

The LD_{50} test and its variants were often undertaken because of legislative requirements. These tests are considered obsolete and nowadays they are rarely but still performed in many countries. Internationally accepted alternative tests have been developed. They use fewer animals and “absence of evident toxicity”, rather than death, as their criterion. They have been accepted by most regulators as valid alternatives to LD_{50} testing. Nonetheless, LD_{50} values are still quoted and enquiries received by the Royal Society of Chemistry suggest that there is still a need for information about their meaning [110]. This was the reason the examination of KLS-10-MA for toxicity to be reported based on LD_{50} test.

The experimental results indicated that all animals from group 2 (healthy and clinoptilolite-supplemented mice) survived up to the end of experiment, gained in body weight, and exhibited a good activity and life condition. No symptoms of an increased toxicity regarding the physiological status were recorded during the experiment. Unfavorable pharmacological effects were not established. In addition, an unusual behavior was not observed. Moreover, the Pb-exposed and supplemented mice demonstrated a significant improvement of the vital parameters. Therefore, due to the lack of any acute toxicity
at the applied dose of the clinoptilolite sorbent, it was impossible to determine LD$_{50}$ of this substance and the sorbent KLS-10-MA was assumed practically nontoxic. However, it is important to note that an increase of sorbent concentration would be wrong because it would worsen the nutrition quality of the animal food.

**Lead bioaccumulation in clinoptilolite unsupplemented and clinoptilolite supplemented mice.**

**Experimental data and model results:** The comparative analysis of lead bioaccumulation between clinoptilolite unsupplemented and clinoptilolite supplemented experimental animals showed substantial differences in these groups (Table 4, Figures 3–6). This fact confirms the usefulness of the clinoptilolite sorbent as a reliable means versus lead intoxication. Below the experimental and modeling results of the investigation is presented and discussed.

The concentration of Pb [mg/kg] in the whole body, liver, and kidneys measured in mice from groups 3 and 4 are presented in Figure 3. The concentration of Pb in bones and feces are displayed in Figures 4 and 5, respectively. Point “0” of the time axis in all of these figures corresponds to the concentration measured in the control group.

The highest Pb concentrations were established in feces, followed by those in bones in the mice from group 3. The background Pb level in carcass, liver, kidney, bones, and feces of the control mice were 0.22 ± 0.06, 0.51 ± 0.07, 0.44 ± 0.08, 0.99 ± 0.15, and 23.6 ± 6.7 mg/kg, respectively. On day 90 the Pb concentrations in carcass, liver, kidney, bones, and feces of the mice group 3 were 1440-fold, 134-fold, 14-fold, and 237-fold, respectively.

The concentration of Pb in bones and feces are displayed in Figures 4 and 5, respectively. Point “0” of the time axis in all of these figures corresponds to the concentration measured in the control group.

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The ratios (7a) and (7b) show that kidney/liver Pb ratio was almost not influenced by the supplementation. Obviously, this fact is related to a strong relationship between liver and kidney Pb levels. 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. This fact clearly indicates 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 decreasing of Pb bioaccumulation in the conditions of the sorbent supplementation:

$$\frac{\text{Pb}_{\text{Bone}}}{\text{Pb}_{\text{Liver}}} = \frac{19}{1}; \quad \frac{\text{Pb}_{\text{Bone}}}{\text{Pb}_{\text{Kidney}}} = \frac{2.5}{1}$$

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The following relations take place:

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

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

Table 4: Mean lead concentrations ± SD (mg/kg) in carcass, liver, kidney, bone, and excreta of the ICR laboratory mice from groups 1 (control), 3 (Pb-exposed), and 4 (Pb-exposed and clinoptilolite-supplemented).
Figure 3: Lead concentrations in carcass, liver and kidneys of ICR mice from groups 3 and 4 during the ecotoxicological experiment. Time point “0” corresponds to the concentrations in the control group.

Figure 4: Lead concentration in bones of ICR mice from groups 3 and 4 during the ecotoxicological experiment – model solutions and experimental points. Time point “0” corresponds to the concentrations in the control group.

Figure 5: Lead concentration in feces of ICR mice from groups 3 and 4 during the ecotoxicological experiment. Time point “0” corresponds to the concentrations in the control group.

Figure 6: The Pb_{90}/Pb_{15} ratio for carcass, liver, kidney, bones, and feces, indicating the significant reduction of Pb bioaccumulation in group 4 versus group 3 due to the clinoptilolite sorbent supplement.
tissues, and feces in the exposed and supplemented animals (group 4). The reduction of 88% of Pb content in the feces of the mice from this group compared to the mice from group 3 indicates that clinoptilolite supplementation can also prevent the contamination of the environment.

The results of the mathematical model are in a good agreement with the experimental data for Pb concentration in bones (Figure 4) and allow determining some parameters of lead-bioaccumulation kinetics.

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

\[ z(t) = z_0 + A_1 a_1 (1 - e^{-b_1 t}) + A_2 a_2 (1 - e^{-b_2 t}) \]

where \( A_1 = a_1 + a_2 \) and \( b_1 = \frac{1}{\eta_1} \), \( b_2 = \frac{1}{\eta_2} \).

The solution \( z(t) \) represents the process of Pb bioaccumulation in the mouse’s bones. Graphically, the time course of bone Pb is presented in Figure 4. The initial condition \( z(0) = z_0 \) 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 \( \eta = 15\% \) [73], 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 \( \eta (0 = \eta = 1) \) is a dimensionless coefficient indicating what fraction of ingested metal dose is resorbed by 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, the value \( A_{\text{Group3}} = a x(t) = 1953 \) mg/kg was obtained. The parameters were fitted by minimizing of \( \chi^2 \) by the use of an iterative Gauss-Newton procedure [111,112]. Thus, the following values were found – for group 3: \( a_1 = 0.022 \) day\(^{-1} \), \( a_2 = 0.001 \) day\(^{-1} \), \( a_3 = 0.004 \) day\(^{-1} \); for group 4: \( A_{\text{Group4}} = x(t) = 459.6 \) mg/kg, \( a_1 = 0.022 \) day\(^{-1} \), \( a_2 = 0.002 \) day\(^{-1} \), \( a_3 = 0.009 \) day\(^{-1} \), and \( a_4 = 0.004 \) day\(^{-1} \).

The model shows that the rate constants of Pb excretion by the feces \( a_3 \), \( a_4 \), is 2-fold higher than \( a_2 \). This is in accord with the real situation. The clinoptilolite sorbent accelerates the intestine passage, as a ballast matter.

This model allowed determining the coefficient of Pb gastrointestinal absorption in the experimental animals from group 4, using the formula:

\[ h = \frac{A_{\text{Group4}} P}{100 B T} \] (10)

where \( h = x(t) \), \( A_{\text{Group4}} = x(t) \), \( P \) is the daily dose of Pb per animal, and \( T \) is the duration of the experiment. The absorption coefficient \( \eta = 3.53\% \) was calculated for the clinoptilolite supplemented mice (group 4). The value \( \eta = 3.53\% \) is 4.25-fold lower compared with \( \eta = 15\% \) in unsupplemented animals. Therefore, KLS-10-MA diminishes the Pb absorption more than four times. The obtained result is very important! It shows a reduction of 76% in Pb quantity entering from the digestive tract to the blood of the mouse organism! This result allows prognosticate an excellent perspective for the application of the clinoptilolite sorbent in mammals.

Genotoxic effects in clinoptilolite unsupplemented and clinoptilolite supplemented mice

Chromosomal aberrations, involving gross alterations of genetic material, have been considered as a sensitive endpoint for detecting genotoxic effects induced by heavy metal and toxic chemicals. Thus, the study of cytogenetical status is considered highly relevant in the human context [113,114].

The chromosome aberration frequency (CAF) in the analyzed metaphases of bone marrow cells of ICR mice in our experiment is presented in Figure 7. 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 \( (\text{CAF}_3) \) exhibited on average 2-fold higher level compared to group 4 and 3.5-fold higher level compared to the control group. The maximum CAF in groups 3 and 4 were observed on day 60. For day 60 the following relations were established:

\[ \text{CAF}_i / \text{CAF}_i = 4.56; \text{CAF}_i / \text{CAF}_i = 2; \text{CAF}_i / \text{CAF}_i = 2.3 \] (1)

The distribution of the different type chromosome aberrations (CA) is displayed in Fig. 8. Chromosome breaks (CrB) and Robertsonian translocations or c/c fusions (C) in group 3 had their maximum values on day 15 (4.7% and 5%, respectively). Chromosome breaks in group 4 were almost equal on days 15 and 90, where they had maximum values (1.75% and 1.5%, respectively). Robertsonian translocations in group 4 had maximum value on day 60 and at this time point groups 3 and 4 showed equal values (4.5%). In all, fragments (F) were found below...
Structural chromosome aberrations (CA) may be induced mainly by direct DNA breakage, by replication on a damaged DNA template, and by inhibition of DNA synthesis [116,117]. They can be divided into two main classes: chromosome-type aberrations (CSA) involving both chromatids of one or multiple chromosomes and chromatid-type aberrations (CTA) involving only one of the two chromatids [117,118]. Metaphase analysis provides information on the timing of DNA lesions relative to DNA replication [119]. CSA result from double-strand breaks (DSB), incompletely- or non-repaired, formed in G1/G0 phase of the cell cycle or from DSB generated before replication in early S phase. In this case, in metaphase are seen chromosome-type breaks, dicentric and ring chromatides, fragments. DSB generated in postreplicative DNA in later S phase and in G2 phase give rise to CTA, i.e. chromatid type breaks and exchanges [118]. CTA may arise also, in response to single-strand breaks (SSB) induced in early S phase. CTA are usually generated by S-phase-dependent clastogens (e.g. chemicals) [120]. SSB resulting from Pb influence were reported by [101,121]. Chromatide breaks observed in metaphase would result from incomplete or failed repair [106]. The high presence of chromatid breaks and Robertsonian translocations in the examined laboratory mice suggested an essential prevalence of CTA. Thus, the data of our experiments, compared with the data of other authors, provide evidence that Pb is mainly S-phase-dependent clastogen.

In Figure 8 it is seen that among the spontaneous CA in mice, centromere–centromeric fusions (c/c fusions) predominate. The mouse karyotype is a special one in some extent because of the acrocentric nature of mouse’s chromosomes. Robertsonian translocations cause structure forms as meta- and sub-meta-centric chromosomes through centric exchanges of two acrocentrics due to the single strand’s breaks in the minor SAT DNA located in the centromeric regions of mice chromosomes. Thus, the rate of exchanges between non-homologous chromosomes in the mouse is particularly high [121].

There is a lack of data about the behavior and adsorption capacity of zeolites in animal organism as well as about the influence of these minerals on genetic apparatus [122] have established that erionite fibers could cause cytogenetic changes similar to those caused by asbestiform mineral dusts but like asbestos minerals do not induce mutations in human lymphoblastoid cells.

Our finding (Figures 7 and 8) showed that clinoptilolite did not give rise to injury of chromosomal structure. The CAF and the percentages

1.5%. Only in group 3 on day 60 F showed a relatively higher value (3%).

The following ratios were established about chromosome breaks, Robertsonian translocations, and fragments between groups 3 and 4 (the time points are given in brackets):

\[
\begin{align*}
CrB_1 / CrB_4 &= 2.7 \ (15), \ CrB_1 / CrB_4 = 7.46 \ (45), \ CrB_2 / CrB_4 &= 12 \ (60) \\
CrB_1 / CrB_2 &= 2 \ (90) \\
C_3 / C_4 &= 1.8 \ (15), \ C_3 / C_4 &= 1.32 \ (90) \\
F_1 / F_2 &= 12 \ (60)
\end{align*}
\]

(2)

The following ratios were calculated regarding the chromatid breaks in a comparison of the experimental groups with the control group (group 1):

\[
\begin{align*}
CrB_1 / CrB_1 &= 4.7 \ (15), \ CrB_1 / CrB_1 = 3.2 \ (45), \\
CrB_1 / CrB_3 &= 3 \ (60) \\
CrB_3 / CrB_3 &= 3 \ (90) \\
CrB_1 / CrB_4 &= 1.8 \ (15), \ CrB_1 / CrB_4 &= 0.43 \ (45), \\
CrB_1 / CrB_2 &= 0.25 \ (60) \\
CrB_2 / CrB_2 &= 1.5 \ (90)
\end{align*}
\]

(3)

To study the proliferative activity of the bone marrow cells during the experiment, mitotic indices were determined in ICR mice (Figure 9). The observed mitotic indices exhibited no statistically significant differences \((p > 0.1)\) between the control and second control groups (groups 1 and 2). Significant differences \((p < 0.05 - p < 0.001)\) were found between groups 3 and 4, between the control group and group 4 \((p < 0.002)\), and especially between the control group and group 3 \((p < 0.001)\). A trend of continuous decrease of the mitotic index during the experiment was established in the exposed-unsupplemented mice. Contrariwise, in the exposed-supplemented mice, the level of the mitotic index decreased up to day 45 but then increased steadily. The following comparisons between the groups were made and respective ratios calculated regarding the mitotic indices \((M)\) (the time points are given in brackets):

\[
\begin{align*}
M_1 / M_1 &= 1.8 \ (15), \ M_1 / M_1 = 2.5 \ (45) \\
M_1 / M_3 &= 2.7 \ (60), \ M_1 / M_3 = 2.9 \ (90) \\
M_1 / M_4 &= 1.4 \ (15), \ M_1 / M_4 = 1.5 \ (45) \\
M_1 / M_2 &= 1.3 \ (60), \ M_1 / M_2 = 1.2 \ (90) \\
M_2 / M_4 &= 1.3 \ (15), \ M_2 / M_4 = 1.6 \ (45) \\
M_1 / M_5 &= 2.1 \ (60), \ M_1 / M_5 = 2.5 \ (90)
\end{align*}
\]

(4)

(5)

In our experiment, the increased percentage aberrant bone marrow cells in the mice from group 3 most probably is due to the very high \(\text{Pb}\) concentration in bones (over 1500 mg/kg on day 90), as seen in Table 4. Lead concentration in bone marrow is proportional to that in bones [115]. Lead is well studied as a factor damaging the chromosome structure in mammalian cells [97,99,115].

Structural chromosome aberrations (CA) may be induced mainly by direct DNA breakage, by replication on a damaged DNA template,
several chromosome aberrations in group 2 exhibited no significant changes compared to those in the control group ($p < 0.1$). The slight increase of CAF on day 90 was almost within the range of spontaneous frequencies. There were no statistically significant differences ($p > 0.1$) in the mitotic indices of the control mice and healthy mice supplemented with clinoptilolite.

The excellent quality end effective influence of KLS-10-MA as a Pb-adsorber in the mouse’s organism is seen from equations (1) – (6) for the chromosome aberrations and (7) – (9) for the mitotic indices. The CAFs in group 4 were 2–2.3-folds lower than those in group 3. The chromatide breaks and fragments in group 4, especially on day 60, were 12-fold lower than those in group 3. On day 90, the mitotic index in group 4 was 2.5-fold higher compared to that in group 3. On the same day the mitotic index in control group was almost 3-folds higher than that in group 3, and only 1.2-fold higher than that in group 4.

These results perfectly conform to the data of Pb bioaccumulation (Table 1). Lead concentration in kidney gives information concerning the level of blood Pb. On day 90, the mean kidney Pb concentration in group 4 was 11-fold lower than that in group 3. Respectively, on day 90 the Pb concentration in bones of the mice from group 4 was 4.3-fold lower compared to Pb bone concentration in mice from group 3. This means that the lead entering into the blood, organs, and bone marrow of the sorbent-supplemented mice was significantly reduced due to the high ion exchange capacity of clinoptilolite in the animal’s digestive tract.

**Pathology in the erythrocytes and erythropoiesis in clinoptilolite unsupplemented and clinoptilolite supplemented mice**

Lead influence on erythropoietic bone marrow cells causes morphological changes, which lead to production of microcytic and hypochromic erythrocytes [94]. 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 with hemoglobin [11]. The increased mechanical fragility of cell membrane shortens the erythrocyte lifespan. Depression of the hemosynthesis by Pb is due to the inhibition of SH-containing enzymes controlling it in bone marrow [11,73]. 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, a well-known symptom of chronic Pb poisoning. 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 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 are presented in Figure 10. 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, also 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 the normal (N) and pathological (P) erythrocytes within groups 3 and 4, on days 15 and 90, respectively:

- group 3: $N_i / P_i = 1.9$ (15), $N_i / P_i = 0.7$ (90) (10)
- group 4: $N_i / P_i = 3.1$ (15), $N_i / P_i = 1.3$ (90) (11)

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

- $N_i / N_i = 2$, $N_i / N_i = 1.35$ (12)
- $P_i / P_i = 0.43$, $P_i / P_i = 0.53$ (13)

Equations (12) and (13) demonstrate a well-expressed toxicological stress in the animals exposed to Pb and a reliable detoxification effect of the clinoptilolite sorbent taken as a food supplement. In the supplemented mice from group 4 pathological erythrocytes were reduced and the normal ones were enhanced so that their levels came near to that in the control group.

Between groups 3 and 4 the following ratios were determined:

- normal Er: $N_i / N_i = 1.2$ (15), $N_i / N_i = 1.5$ (90) (14)
- pathological Er: $P_i / P_i = 1.4$ (15), $P_i / P_i = 1.2$ (90) (15)

Equations (14) and (15) confirm the essential effect of the sorbent on the decrease of harmful effect of Pb on cell level.

A significant drop in the erythropoiesis rate was recorded in the Pb-exposed mice. The time course of the proliferating erythrocytes (PE) is presented in Figure 11. Statistically significant differences ($p < 0.05$) were observed on days 15 and 90 between groups 3 and 4. The percentages proliferating erythrocytes in Pb-exposed and supplemented mice were higher than those in Pb-exposed mice without clinoptilolite supplement. The differences between groups 3 and 4, and control group 1, especially on day 90 were quite significant ($p < 0.001$). Comparing PE in the control mice to that in mice from groups 3 and 4 on day 90 the following ratios were obtained:

- $PE / PE = 10$, $PE / PE = 3.2$ (16)

The following ratios between PE in group 4 and PE in group 3 taken place:

- $PE / PE = 1.22$ (15), $PE / PE = 3.14$ (90) (17)

A 10-fold drop was observed in the number of dividing erythroblasts in group 3 compared with the control group (equation (16)). The supplementation with KLS-10-MA reduced the respective ratio in group 4 almost by three times. The greatest differences between groups 3 and 4 were established on day 90, equation (17). The result indicates that the sorbent improved the erythropoietic function after day 60.

No many echinocytes were found and this could be due to the relatively low Pb level in the liver (compared to other tissues); on day 90. In group 3 it was 68.5±18 mg/kg (Table 1). The echinocytes presence mainly in group 3 suggests that the potassium (K) in the mouse’s organism is replaced in high degree by Pb. The lack of echinocytes in the mice from group 4 indicates a normal K level. In fact, on day 90 the mean liver Pb concentration in group 4 was almost 8-fold lower than in group 3 (Table 4) and therefore, the amount of replaced K in group 4 was insignificant. Moreover, the supplemented mice have received additional K from the sorbent. The observed data are in accord with the finding of [123] who detected a 20% elevation of serum K in healthy laboratory mice receiving a zeolite-rich diet.

Micronuclei in erythrocytes in peripheral blood were observed in the specimens from group 3 and 4. In group 4 they appeared sporadically mainly before day 45. This fact indicates again that the main part of Pb entering in the mouse organism via drinking water was captured by the clinoptilolite sorbent and thus the Pb quantity absorbed by the intestine mucosa and utilized by the organism at cell level was significantly reduced.

**Growth pattern in clinoptilolite unsupplemented and clinoptilolite supplemented mice**

Body weight gain in experimental animals is a reliable indicator for their healthy status. It is an integral characteristic of the physiology of the organism. In the experiment here presented the body weight gain of the mice was determined at the same time points, in which the samples for the cytogenetical and hematological analyses were taken: on days 15, 45, 60, and 90.

The changes in the body weight of the mice are presented in Fig. 12. Statistically significant differences were found among groups 3 and 4 ($p < 0.01$) on days 15, 45, 60 and 90 ($p < 0.01$). A sharp decrease of body weight was established in the Pb-exposed mice from group 3 after day 60. On day 90 these mice exhibited a body weight reduced with 24% compared to the control mice. On day 90 the body weight 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 the control group during the experiment.

When compared the body weight ($W_{90}$) on day 90 to the body weight ($W_{0}$) on day 90 in the mice from groups 3, 4 and 1 (control) the following ratios were calculated:

**Figure 11: Percentage of proliferating erythrocytes in ICR mice.**

![Figure 11: Percentage of proliferating erythrocytes in ICR mice.](image-url)
The positive effect of the clinoptilolite sorbent KLS-10-MA on the supplemented mice confirms the numerous data of other authors who have observed essential positive results, such as toxin elimination, conversion nutrition improvement, growth increase, mortality reduction, as well as improvement of common state of animal health using clinoptilolites as a feed additive in poultry, pigs and ruminants nutrition [17].

Studies illustrate that clinoptilolite supplementation to the diet of swine, poultry and ruminants improved body mass and feed conversion ratios [107,124,125]. In addition, milk yields have improved in dairy herds and the incidence of scour, enteritis and other intestinal diseases has decreased substantially [107]. The use of 3%, 5% and 10% clinoptilolite as a feed supplement for Leghorn chickens, indicated that at all levels, feed efficiency ratios have increased comparing with the control diet. No adverse effects on the health or vitality of the birds have been observed [107]. Clinoptilolite at 2% supplement level by weight in a hog diet increased daily feed intake [126,127]. A significant daily gain of newborn lambs was established in the conditions of basal diet with 3% clinoptilolite [128]. A greater weight gain has been reported of 17% and 3.6% in young beef cattle and broilers when clinoptilolite at 4.3% and, 0.5%, 1% and 1.5% by weight was added to cattle’s and chickens’ regular diet, respectively [129]. In comparison to a regular diet, the clinoptilolite at 5% and 10% levels by weight in hog’s diet resulted in a weight gain of 27% [130-134] and 8% [129], respectively.

In the present experiment the modified clinoptilolite KL-10-MA was mixed at 12.5% concentration with the conventional forage. Therefore, approximately about 9% by weight was added to the mice’s daily diet. The data showed a 5% rise (Figure 12) in body weight of the healthy supplemented animals (group 2) compared with the healthy non-supplemented ones (control group). Comparing the obtained results with the data of other authors, the optimal quantity of clinoptilolite supplement ensuring the maximal body weight gain could be specified. Perhaps there should be doses of about 5% by animal weight.

**Conclusion**

The chromosome aberrations, lowered mitotic index, pathologically changed erythrocytes, diminished erythropoiesis, and reduced body weight gain observed here in the Pb-exposed laboratory mice, demonstrated a well-expressed toxicological stress due to the chronic
Pb intoxication. The structure of chromosomes and red blood cells as well as mitotic index and erythropoiesis were significantly improved by the supplementation of the animals with modified clinoptilolite sorbent KLS-10-MA, a Na-enriched alcali earth clinoptilolite based on natural Bulgarian clinoptilolite. 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 bioaccumulation coefficients \( \text{Pb}_{\text{b}} / \text{Pb}_{\text{s}} \) for carcass, liver, kidney, bones, and feces in supplemented animals were significantly lower compared to those in unsupplemented ones. Thus, the obtained results showed that the sorbent KLS-10-MA strongly decreases the absorption of lead in animals’ digestive tract and therefore limits Pb quantity entering the blood. The time course of the body weight in the supplemented animals differed not significantly from that in the control mice. The supplemented animals survived and appeared active and healthy. A weak rise of the body weight gain was established in healthy clinoptilolite-supplemented animals.

The mathematical model of Pb bioaccumulation in bones can predict the time course of Pb concentrations in conditions of chronic exposure to Pb with/without sorbent supplementation. The aim of the model is to indicate the main tendencies of Pb bioaccumulation in bones, taking into account the basic factors determining this process. The model allows determining some kinetic parameters, especially, it was useful to calculate the coefficient of Pb gastrointestinal absorption in the case of sorbent-supplemented animals. This coefficient was 4 times lower compared to the established coefficient for Pb absorption.

The findings allow conclude that the clinoptilolite sorbent KLS-10-MA exerts a significant favorable effect and thus it appears as a reliable means for detoxification of human and animal organisms chronically poisoned by heavy metals, particularly lead. This work gives ground to consider the use of Pb sorbents for detoxification of human and animal organisms chronically poisoned by heavy metals, particularly Pb, in order to protect the animals’ health and the quality of the environment. The use of a drug based on this sorbent could be valuable in agriculture and livestock as well as in human medicine in cases of chronic lead intoxication.

References

1. Sheppard RA (1984) Characterization of zeolitic materials in agricultural research. In: Pond MG, Mumpton FA (eds) Zeo-Agriculture: Use of Natural Zeolites in Agriculture and Aquaculture. Westview Press, Boulder, Colorado 81-90.
2. Mumpton FA (1999) La roca magica: uses of natural zeolites in agriculture and industry. Proc Natl Acad Sci U S A 96: 3463-3470.
3. Inglezakis VJ, Stylianou MA, Gkantzou D, Loizidou MD (2007) Use of natural clinoptilolite for the removal of lead, copper and zinc in fixed bed column. J Hazard Mater 143: 575-581.
4. Papaioannou D, Katsoulos PD, Parousis N, Karatzias H (2005) The role of natural and synthetic zeolites as feed additives on the prevention and/or the treatment of certain farm animal diseases: A review. Micropor Mesopor Mat 84: 161-170.
5. Stylianou MA, Hadjiconstantinou MP, Inglezakis VJ, Moustakas KG, Loizidou MD (2007) Use of natural clinoptilolite for the removal of lead, copper and zinc in fixed bed column. J Hazard Mater 143: 575-581.
6. Orhan Y, Kocaoglu S (2007) Adsorption of toxic metals by natural and modified clinoptilolite. Ann Chim 97: 781-790.
7. Tao YF, Qiu Y, Fang SY, Liu ZY, Wang Y, et al. (2010) Trapping the lead ion in multi-components aqueous solution by natural clinoptilolite. J Hazard Mater 182: 282-288.
8. Sampson R (1997) Application for the approval of clinoptilolite. Euremica Environmental Ltd, Instrument House, Cleveland, UK.
9. Eady SJ, Pritchard DA, Martin MDJ (1980) The effect of sodium bentonite on zootherm on wool growth of sheep fed either mulga (Acacia aneura) or lucerne (Medicago sativa). Proc Aust Soc Anim Prod18: 188-191.
10. Vrzgula L, Bartko P, Blazovsky J, Kozac J (1982) [The effect of feeding clinoptilolite on the health status, blood picture and weight gain in pigs]. Vet Med (Praha) 27: 267-274.
11. Keshavarz K, McCormick CC (1991) Effect of sodium aluminoisolate, oyster shell, and their combinations on acid-base balance and eggshell quality. Poult Sci 70: 313-325.
12. Ward TL, Watkins KL, Southern LL, Hoyt PG, French DD (1991) Interactive effects of sodium zeolite-A and copper in growing swine: growth, and bone and tissue mineral concentrations. J Anim Sci 69: 726-733.
13. Fethiere R, Miles RD, Harms RH (1994) The utilization of sodium in sodium zeolite A by broilers. Poult Sci 73: 118-121.
14. PeriÅ‡ J, Trgo M, VukojeviÄ‡ MedvidoviÄ‡ N (2004) Removal of zinc, copper and lead by natural zeolite-a comparison of adsorption isotherms. Water Res 38: 1893-1899.
15. Mumpton FA (1960) Clinoptilolite redefined. Am Mineral 45: 351-369.
16. Sampson R, Scott KM (1993) Application of natural zeolites for the reduction of ammonia emissions during the composting of organic wastes in a laboratory composting simulator. Bioresource Technol 43: 35-39.
17. Jongbloed AW, Lensis NP (1998) Environmental concerns about animal manure. J Anim Sci 76: 2641-2648.
18. Sutton AL, Kephalt KB, Verstegen MW, Canh TT, Hobbs PJ (1999) Potential for reduction of odorous compounds in swine manure through diet modification. J Anim Sci 77: 430-439.
19. Dwairi IM (1998) Conserving toxic ammonical nitrogen in manure using natural zeolite tuft: a comparative study. Bull Environ Contam Toxicol 60: 126-133.
20. Goyer RA (1996) Toxic effects of metals. In: McGraw-Hill (ed) Cassaret and Doull’s Toxicology. The Basic Science of Poisons. Fifth (edn). Health Professions irvision, New York • London • Tokyo • Singapore 691-735.
21. O’Flaherty EJ (1998) Physiologically based models of metal kinetics. Crit Rev Toxicol 28: 271-317.
22. Robbins HV,romo E, Sanchez-Mendoza A, Rios A, Soto V, et al. (2007) Lead exposure effect on angiogenesis in renal vasoconstriction. Hum Exp Toxicol 26: 499-507.
23. Johnson FM (1998) The genetic effects of environmental lead. Mutat Res 410: 123-140.
24. Topshaka-Ancheva M, Metcheva R, Teodorova S (2003) Bioaccumulation and damaging action of polymetal industrial dust on laboratory mice Mus musculus alba II. Genetic, cellular and metabolic disturbances. Environ Res 92: 152-160.
25. Valverde M, Trejo C, Rojas E (2001) Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? Mutagenesis 16: 265-270.
80. Milton A, Cooke JA, Johnson MS (2003) Accumulation of lead, zinc, and cadmium in a wild population of Cleftirorhynchus glareolus from an abandoned lead mine. Arch Environ Contam Toxicol 44: 405-411.

81. Sindhu RK, Sindhu KK, Roberts CK (2007) Role of lead in hypertension and chronic kidney disease. Environ Res J 1: 345-359.

82. Samuels ER, Meranger JC, Tracy BL, Subramanian KS (1989) Lead concentrations in human bones from the Canadian population. Sci Total Environ 89: 261-269.

83. Hamilton JD, O’Flaherty EJ (1995) Influence of lead on mineralization during bone growth. Fundam Appl Toxicol 26: 265-271.

84. O’Flaherty EJ (2000) Modeling normal aging bone loss, with consideration of bone loss in osteoporosis. Toxicol Sci 55: 171-188.

85. Luckey TD, Venugopal B (1977) Metal toxicity in mammals. 1. Physiologic and biochemical basis for metal toxicity. Plenum Press, New York and London.

86. Mavrodiev S (1999) Applied ecology of the Black Sea. Nova Science Publishers Inc., New York.

87. O’Flaherty EJ, Inskip MJ, Franklin CA, Durbin PW, Manton WI, et al. (1998) Lead and aluminium in mouse bone marrow cells by dietary ingestion of lead nitrate. Acta Anat (Basel) 135: 185-188.

88. O’Flaherty EJ, Insipk MY, Jagminas AP, Franklin CA (1996) Plasma and bone lead concentrations, lead absorption, and lead excretion in nonhuman primates. Toxicol Appl Pharmacol 138: 121-130.

89. O’Flaherty EJ, Inskip MJ, Franklin CA, Durbin PW, Manton WI, et al. (1998) Evaluation and modification of a physiologically based model of lead kinetics using data from a sequential isotope study in cynomolgus monkeys. Toxicol Appl Pharmacol 149: 1-16.

90. Fowler BA, Kimmel CA, Woods JS, McConnell EE, Grant LD (1980) Chronic low-level lead toxicity in the rat. Ill. An integrated assessment of long-term toxicity with special reference to the kidney. Toxicol Appl Pharmacol 56: 59-77.

91. Fowler BA, Taylor JA, Oskarsson A (1981) Compartmental binding of Pb in rat kidney mitochondria. Fed Proc 40: 828-830.

92. Hegglund M (1975) Differential diagnosis of internal diseases. Georg Thieme Verlag, Stuttgart.

93. Cooke JA, Johnson MS (1996) Cadmium in small mammals. In: Beyer WN, Heinz GH, Redmon-Norwood AW (Eds) Environmental contaminants in wildlife. Interpreting tissue concentrations. Lewis publishes, Boca Raton New York London Tokyo.

94. O’Flaherty EJ, Adams WD, Hammond PB, Taylor E (1986) Resistance of the rat to development of lead-induced renal functional deficits. J Toxicol Environ Health 13: 61-75.

95. Goyer RA (1971) Lead toxicity: a problem in environmental pathology. Am J Pathol 64: 167-182.

96. Paglia DE, Valentine WN, Dahlgren JG (1975) Effects of low-level lead exposure on pyrimidine-5'-nucleotidase and other erythrocyte enzymes. Possible role of pyrimidine-5'-nucleotidase in the pathogenesis of lead-induced anemia. J Clin Invest 56: 1164-1169.

97. Beltcheva M, Metcheva R, Popov N, Teodorova SE, Heredia Rojas JA, et al. (2012) Natural clinoptilolite detoxifies small mammals’ organism loaded with lead I: Lead disposition and kinetic model for lead bioaccumulation. Biol Trace Elem Res 147: 180-188.

98. Marques CC, Nunes AC, Pinheiro T, Lopes PA, Santos MC, et al. (2006) An assessment of time-dependent effects of lead exposure in Algerian mice (Mus musculus) using different methodological approaches. Biol Trace Elem Res 109: 75-90.

99. Nayak BN, Ray M, Persaud TV (1989) Maternal and fetal chromosomal aberrations in mice following prenatal exposure to subembryotoxic doses of lead nitrate. Acta Anat (Basel) 135: 185-188.

100. Dhir H, Roy AK, Sharma A, Talukder G (1990) Modification of clastogenicity and chromosomal breaks from progressing to chromosomal breaks and translocations. DNA Repair (Amst) 5: 1030-1041.

101. Hartwig A, Schlepegrell R, Beyersmann D (1990) Indirect mechanism of lead-induced genotoxicity in cultured mammalian cells. Mutat Res 241: 305-312.

102. Topashka-Ancheva M, Metcheva R, Teodorova SE (2003b) A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: chromosomal aberrations and blood pathology. Ecotoxicol Environ Safety 54: 188-193.

103. Silbergeld EK, Wалалkes M, Rice JM (2000) Lead as a carcinogen: experimental evidence and mechanisms of action. Am J Ind Med 38: 315-323.

104. Beyermann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82: 493-512.

105. Roy NK, Rossman TG (1992) Mutagenesis and cometogenesis by lead compounds. Mutat Res 298: 97-103.

106. Topashka-Ancheva M, Beltcheva M, Metcheva R, Heredia Rojas JA, Rodríguez De la Fuente AG, et al. (2012) Modified natural clinoptilolite detoxifies small mammal’s organism loaded with lead II. Genetic, cell, and physiological effects. Biol Trace Elem Res 147: 206-216.

107. Pirrelli P, Goeckede W, Obe G (2000) Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis 15: 209-302.

108. Mumpton FA (1985) Using zeolites in agriculture in innovative biological technologies for less developed countries. Workshop Proceedings. Washington DC.

109. Mumpton FA (1983) The role of natural zeolites in agriculture and aquaculture. In: Pond WG and Mumpton FA (Eds) Zeo-Agriculture: Use of Natural Zeolites in Agriculture and Aquaculture. Westview Press, Boulder, Colorado.

110. Pond WG, Kroop LP, Ho H, Su D, Schoknecht PA (1996) Bone density and tissue lead accretion in growing rats fed low high calcium with or without supplemental clinoptilolite. Bull Environ Contam Toxicol 57: 713-721.

111. Environment, Health and Safety Committee [EHC] (2001) Note On “LDSO” [LETHAL DOSE 50%]. Version 2 – 31/7/01. Royal Society of Chemistry, London.

112. Alexandrov L (1971) Regularized computerizing processes of Newton-Kantorovich type. JVM I MF 2; 31-41 (In Russian).

113. Alexandrov L, Drenska M, Karadjov D (1984) Program System REGN for solution of nonlinear system of equations PRS-165/REGN RSIC. Osak Ridge, Tennessee.

114. International Commission for Protection Against Environmental Mutagens and Carcinogens (1983) Report of Committee I. Mutation Res 114: 120-177.

115. World Health Organization (1985) Environmental Health Criteria 5. Guide to Short-term Tests for Detecting Mutagenic and Carcinogenic Chemicals. WHO, Geneva.

116. Dernarque D, Jouanny J, Poltevin S, Saint Jean Y (1995) Pharmacology and Homeopathic Medical Matter. Boiron- C.E.D.H., France.

117. Sorsa M, Wilbourn J, Vainio H (1992) Human cytogenetic damage as a predictor of cancer risk. In: Vainio H, Magee PH, McGregor DB, McMichael AJ (Eds) Mechanisms of Carcinogenesis in Risk Identification). Lyons & International Agency for Research on Cancer.

118. Albertini RJ, Anderson D, Douglas GR, Hageman L, Hemminki K, et al. (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. Mutat Res 463: 111-172.

119. Hagnar L, Strömberg U, Bonassi S, Hansteen IL, Knudsen LE, et al. (2004) Increased risk: results from Nordic and Italian cohorts. Cancer Res 64: 2258-2263.

120. Franco S, Alt FW, Manis JP (2006) Pathways that suppress programmed DNA repair (Amst) 5: 1030-1041.

121. Mateuca R, Lombaert N, Aka PV, Devordier I, Kirsch-Volders M (2006) Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie 88: 1515-1531.

122. Kipling D, Wilson HE, Mitchell AR, Taylor BA, Cooke HJ (1994) Mouse centromere mapping using oligonucleotide probes that detect variants of the centromere's alpha satellite. Chromosoma 103: 46-55.

123. Kelsey KT, Yano E, Liber HL, Little JB (1986) The in vitro genetic effects of fibrous erionite and crocidolite asbestos. Br J Cancer 54: 107-114.

124. Martin-Kleiner I, Flegar-Mestric Z, Zadro R, Breijak D, Stanovic Janda S, et al. (2001) The effect of the zeolite clinoptilolite on serum chemistry and hematopoiesis in mice. Food Chem Toxicol 39: 717-727.
125. Shurson GC, Ku PK, Miller ER, Yokoyama MT (1984) Effects of zeolite a or clinoptilolite in diets of growing swine. J Anim Sci 59: 1536-1545.

126. McComb E, Brewster P, Chou PH, Crossley M, Simard M, et al. (2010) Telephone administration of the Mental Alternation Test: sensitivity to cognitive decline and practice effects across midlife and late life. Neuroepidemiology 35: 298-302.

127. Pond WG, Yen JT (1988) Response of growing pigs to dietary clinoptilolite from two geographic sources. Nutrition Reports International 25: 837-848.

128. Yannakopoulos A, Tserveni-Gousi A, Kassoli-Four-Naraki A, Tsirambides A, Michailidis K, et al. (2000) Effects of dietary clinoptilolite-rich tuff on the performance of growing-finishing pigs. In: Coela C, Mumpton FA (Eds) Natural zeolites for the third millennium. De Frede Editore, Napoli, Italy.

129. Norouzian MA, Valizadeh R, Khadem AA, Afzalzadeh A, Nabipour A (2010) The effects of feeding clinoptilolite on hematology, performance, and health of newborn lambs. Biol Trace Elem Res 137: 168-176.

130. Nestorov N (1984) Possible applications of natural zeolites in animal husbandry. In: Pond WG, Mumpton FA (Eds) Zeo-Agriculture: Use of Natural Zeolite in Agriculture and Aquaculture. International committee on natural zeolites. Westview Press, Boulder.

131. Kondo N, Wagai B (1968) Experimental use of clinoptilolite-tuff as a dietary supplement for pigs. Yotonkai.

132. Ming DW, Dixon JB (1987) Quantitative determination of clinoptilolite in soils by a cation-exchange capacity method. Clays and Clay Minerals 35: 463-468.

133. Privezentsev KV, Sirota NP, Gaziev AI (1996) [The genotoxic effects of cadmium studied in vivo]. Tsitol Genet 30: 45-51.

134. Zhou CF, Zhu JH (2005) Adsorption of nitrosamines in acidic solution by zeolites. Chemosphere 58: 109-114.