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Does Natural Clinoptilolite Induce Toxicity in Small Mammals?*

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Abstract: The study aimed to assess the safety of using modified natural clinoptilolite as a food additive for mammals. Any potential adverse impacts of altered natural clinoptilolite on the regular physiological processes of animals were evaluated. Sixty male ICR laboratory mice, aged approximately 6–8 weeks, were randomly assigned to control and experimental groups. Both groups underwent a 45-day testing period, during which the experimental group was given rodent food mixed with 12.5% modified natural clinoptilolite from the Beli Plast deposit, Bulgaria. Sampling was carried out on days 0, 15, 30 and 45, with each time point providing samples for morpho-physiological and haematological studies, the oxidative stress on the base of malondialdehyde (MDA) and glutathione (GSH) measurements in liver and kidney so as the assessment of gene toxicity via the micronucleus test. All animals used in the experiment survived until the end of the study, gained weight and showed good activity and vital signs. The mice supplemented with clinoptilolite showed 21% higher body weight gain compared to the control group. Furthermore, there were no established signs of gene toxicity, pathological changes in the levels of oxidative stress and haematological parameters. These results suggest that using clinoptilolite as a food supplement does not lead to observable toxicities and even improves growth performance. Owing to its elevated sorption potential, this method is appropriate for purging the mammalian organism of toxins, with negligible adverse outcomes.

Key words: clinoptilolite, mice, haematology, gene toxicity, oxidative stress

Introduction

Zeolites, especially clinoptilolites, are crystalline, hydrated aluminosilicates of alkali and alkaline earth cations with an infinite, open, three-dimensional structure. They are capable of reversible water loss and gain as well as the exchange of additional cations, all while maintaining their original crystal structure (Mumpton 1999). Depending on their structure, mineralogical and chemical charac-

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teristics, natural zeolites exhibit several properties directly related to their applications as food supplements or body detoxifiers. The main properties of zeolite in this aspect are (1) alkaline nature, (2) cation exchange, (3) physical sorption and (4) highly pronounced surface activity. This main characteristic of clinoptilolite related to its sorption properties and the elimination of toxicants, which is practically a support for the "homeostasis of the body", can find many applications. The application of natural zeolites as food additives for animals started in Japan in the mid-1960s initially with swine and poultry, and later expanded to other animals. The findings were highly satisfactory and indicated accelerated development alongside enhanced productivity (Torii 1974, Torii 1978, Mumpton 1978). The administration of natural zeolite also resulted in a decreased occurrence of diseases and mortality in livestock, leading to suggestions of its prophylactic potential. Numerous studies have demonstrated the positive impact of clinoptilolite on both growth and productivity in the field of animal husbandry as well as its ability to enhance the sorption capacity of the mineral during detoxification processes in living organisms. In Bulgaria, sizable deposits of high-quality zeolites are present, which are majorly composed of clinoptilolite (Alexiev & Djourova 1975). There are six commercial potential zeolite deposits present in the North-Eastern Rhodopes region, including "Beli Plast", "Gorna Krepost", "Most", "Golobradovo", "Lyaskovets" and "Beliya Bair". These deposits have the potential to serve as valuable resources for future applications, particularly as effective detoxifiers. Insufficient data exists regarding the presence of any toxicity of the zeolite in animals' bodies and, thus, the safety of *in vivo* administration of modified natural clinoptilolite as a food additive has not been fully established. Based on current scientific knowledge, it is hypothesized that modified natural clinoptilolites do not display any toxic effects, although further research is necessary to verify this assumption. The first studies in Bulgaria regarding the *in vivo* sorption capacity of modified natural clinoptilolite in laboratory white mice are for chronic lead intoxication (Beltcheva et al. 2012, Topashka-Ancheva et al. 2012, Beltcheva et al. 2015). The positive outcomes and the wide professional acceptance of the published data suggest that the amplification of research in this field would promote substantial advancement. It is the subject of the current study to determine the composition, biological effects and biological safety of modified natural clinoptilolite, specifically those sourced from the "Beli Plast" deposit. Determination of possible lethal dose as well as changes in body mass, basic hematological parameters, oxidative stress levels and DNA damages caused by micronuclei presence in erythrocytes were evaluated on ICR laboratory mice, which are the suitable model organisms.

Materials and Methods

Experimental design

The tested animals were divided into two groups: the control group where mice were fed with standard food for small rodents and the zeolite-supplemented group, where standard food was mixed with 12.5 wt. % modified natural clinoptilolite. Food was offered in the form of pellets. All mice were allowed access to food and water *ad libitum*. Mice were acclimatized for 7 days before starting the experiment. Each experiment lasted 45 days. Samples were collected from each experimental group on days 0, 15, 30 and 45. The body mass of each animal was recorded, blood samples were taken from the tail vein and the kidneys and liver were dissected for further analysis. A standard temperature between 19°C and 23°C, humidity of 45–60% and a 12-hour day/night cycle were maintained throughout the study. The water, food and bedding materials were inspected daily and changed when necessary.

Clinoptilolite sorbent

The clinoptilolite-rich tuff was collected from the volcanogenic sedimentary deposit "Beli Plast" (Eastern Rhodopes, Bulgaria), related to the first Rupelian acid volcanic phase that occurred at the beginning of the Oligocene. During the formation of the deposit in a marine environment, the metastable volcanic glass was transformed into more stable phases, which are dominated mainly by clinoptilolite (Yanev et al. 2006). The clinoptilolite-rich tuff was subjected to grinding and sieving and the fraction below 0.15 mm was separated for treatment. Clinoptilolite was exchanged into its sodium form by stirring 100 g of the natural sample and 1000 ml of 1 M $NaNO_3$ solution at 500 rpm. The treatment was performed in a closed bottle placed in an oven heated at 80 °C. The $NaNO_3$ solution was replaced every day by a fresh one. This procedure was repeated 12 times. The sample was washed with deionized water to remove the occluded salt and was then dried at 60 °C in an oven.

The mineral composition of the zeolitised tuff was determined by powder X-ray diffraction (PXRD). The PXRD patterns were collected at room temperature on a PANalytical EMPYREAN Diffractometer system with a goniometer radius of 240 mm, operating at 40 kV and 30 mA, with CuKα radiation, equipped with a 3D-pixel detector. PXRD measurements were recorded in the 2θ range 3–70° with a scanning rate of 0.013° for 80 s. Major element contents (Si, Ti, Al, Fe, Mn, Mg, Ca, Na, K and S) both in the initial and modified samples were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The trace element content of the zeolitised tuff was determined by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) using a PerkinElmer ELAN DRC-e ICP-MS equipped with a New Wave UP193-FX excimer laser system (GI–BAS). Fused pellets with lithium tetraborate were prepared at 1050°C in Pt crucibles. The concentrations for the sample are the average values of the three measurements done on the pellet.

Experimental animals

Fifty-six males, 6–8 weeks old laboratory mice of the ICR strain were used in all experiments. They were bred in a dedicated vivarium and housed in individually ventilated cages by European standards (Directive 2010/63/EU on the protection of animals in science). The bedding material was obtained from an ISO 2000-accredited supplier. The rearing of animals in a vivarium and the conduct of experiments are in accordance with Ordinance No. 20/01.11.2013 on the Protection and Welfare of Animals and the Animal Protection Act (31.01.2008) of the Republic of Bulgaria. During sampling, the team used a maximum of animal-friendly, low-invasive and non-invasive methods, in full compliance with the OECD Guidelines of 17.12.2001 and 29.07.2016.

Morpho-physiological parameters and haematology

The body mass of the experimental animals and growth performance were recorded at each experimental time point. Morpho-physiological analysis was carried out according to the method of liver and kidney index (Cattley & Cullen 2013), which was calculated as the ratio of organ weight to body weight, expressed per mille according to the following formula:

*Organ mass index = (Organ Mass / Total body mass) * 1000*,

which gives a standard index expressed in mg/g body mass.

Haematological analysis was performed on a Mindray BC-30Vet haematology analyser. The following haematological indices were recorded: erythrocytes parameters (RBC – red blood cell count, HGB – haemoglobin concentration, HCT – haematocrit value, MCV – mean corpuscular volume of erythrocytes), platelets (PLT) and white blood cell parameters (WBC – total number of the white cells and differential blood cell scoring – Gr granulocytes, Mo monocytes, etc.).

Oxidative stress determination (MDA + total glutathione)

Oxidative stress was measured in the liver and kidney of experimental and control animals at each time point. Two markers for oxidative stress were selected:

1) The relative level of malondialdehyde (MDA) and other lipid peroxidation products in organ homogenates measured by the thiobarbituric acid reactive substances (TBARS) test as a biomarker for lipid degradation of reactive oxygen species ROS).

2) The relative amount of total glutathione in the same homogenates (as a biomarker of defence against oxidative stress).

The TBARS was performed according to de Leon & Borges (2020). Mouse liver and kidney were weighed, homogenized in ice-cold trichloroacetic acid (TCA) and subjected to the standard protocol. The levels of MDA and the total glutathione were measured colorimetrically and expressed as μmol/g fresh weight. Total GSH was assayed using Ellmann's reagent (Şehirli et al. 2008). All photometry was performed on a BOECO S-22 UV/Vis spectrophotometer.

***In vivo* micronucleus test**

A gene toxicity screening procedure was conducted on 56 mouse peripheral blood smears via the *in vivo* micronucleus test. Acridine orange fluorescence staining was performed using a standard protocol by Hayashi et al. (1983), with Sorensen's phosphate buffer replaced by phosphate-buffered saline (PBS). The slides were visualized using an Optika B-383FL fluorescence microscope (40x/0.65 objective). Cells in which, in addition to the main nucleus, there is a less isolated nucleus with a round or oval shape, which is not larger than 1/5 of the main nucleus and which fluoresces with the same colour as the main nucleus, are considered to be erythrocytes with micronucleus (MN). The mean frequency of MN per 2000 scored erythrocytes (polychromatic and normochromatic), expressed in parts per thousand (per mille), was calculated for each individual using the following formula:

$$MN \text{ frequency ‰} = \frac{\text{Number of cells containing MN}}{\text{Number of cells scored)}} \times 1000$$

All the data were first tested for normal distribution (D'Agostino and Pearson omnibus normality test) and homogeneity of variance (Levene, F-test). As the data passed the normality test ($p < 0.001$), one-way ANOVA followed by Tukey's multiple comparison post-test and two-tailed t-test were used. For all tests, the significance level was set at $p \leq 0.05$. All calculations were performed using the Prism software package, version 9 (GraphPad Software, San Diego, CA, USA).

Results

Clinoptilolite tuff composition

The mineral assemblage of the zeolitic tuff (fraction 0.15 mm) determined by semi-quantitative PXRD analysis included clinoptilolite ~86%, opal-CT ~7%, mica (10 Å) ~2%, montmorillonite ~2% and plagioclase ~2%. The chemical composition of the major elements in wt. % was: 71.08 SiO_2, 0.12 TiO_2, 10.88 Al_2O_3, 0.96 Fe_2O_3(t), 0.09 MnO, 1.03 MgO, 2.78 CaO, 0.31 Na_2O, 2.76 K_2O, 0.15 SO_3 and ignition losses, i.e., adsorbed and chemically bonded water about 11.61. The values of the oxides of the exchangeable cations CaO, K_2O and MgO after Na-exchange decreased and were 1.23, 2.32 and 0.72, respectively. The Na_2O content increased significantly and reached 2.57 wt %.

The contents of 63 trace elements were determined to establish the presence of toxic and potentially toxic elements in the clinoptilolite tuff. Results were compared to those of the Upper continental crust (Rudnick & Gao 2014). The contents of the elements Co, Ge, As, Se Mo, Cd, Te, Eu, Re, Rh, Pd and Au were found to be below the detection limits. The clinoptilolite tuff was relatively depleted in Sc, V, Cr, Ni, Cu, Zn, Ga, Y, Zr, Ba, Hf, W and rare earth elements (REEs) (except for lanthanum, thulium and ytterbium). A slightly enhanced concentration was found for the elements Be, S, Rb, Sr, Nb, In, Sn, Sb, Cs, Ta, Tl, Pb, Bi, Th, U and Ag. According to EU Regulation 744/2012 (EEA 2012), the used clinoptilolite from the "Beli Plast" deposit can be utilized as a feed additive because its content of Pb is below the maximum permissible value of 60 ppm and can serve as a sorbent for detoxification purposes.

The lethal dose of clinoptilolite

During the experiment, LD50 of the tested mineral was tried to be registered. The results showed that all animals fed with zeolite as a food supplement survived through the end of the experiment. Moreover, the animals exhibited increased body mass as well as good activity and vitality during the trial. No signs of toxicity resulting from changes in their physiological state were observed throughout the entire duration of the experiment. Neither pharmacological intoxication nor any unusual behaviour was detected. This study supports that the application of modified clinoptilolite does not exhibit toxicity.

Changes in animals' body mass

No significant differences ($p > 0.05$) in body mass gain were found between the experimental groups during the first two weeks of the experiments. After day 15, both the control and zeolite-exposed groups exhibited weight gain, albeit faster in the group supplemented with zeolite. Significant differences ($d < 0.05$, $t=3.1$, $df=4$) in weight were observed on days 30 and 45, indicating an average body weight difference of approximately 4.0 g (Fig. 1).

Organ mass indices and haematology

Both the control and zeolite-supplemented groups showed normal decreases in relative liver and kidney size during the growth and maturation phase in mice, as expected (Fig. 2). In the control group, liver and kidney indices demonstrated a significant decline ($p < 0.05$) throughout the experiment, with the lowest values recorded on day 45. Conversely, the zeolite-exposed group had a statistically significant decrease in liver and kidney indices only on day 45 (Fig. 2). Significant differences ($p < 0.05$) were detected between the zeolite-exposed group indexes and the control group indexes on day 0. The overall trends in the control and zeolite-exposed groups are similar, without alarming values indicating a physiological change in either of the two groups.

Haematology

The effects of zeolite dietary treatment on haematological parameters are presented in Table 1. The analysis confirmed little observable effects of zeolite exposure. Erythropoiesis data show no statistically significant differences between controls and zeolite-supplemented groups ($p < 0.05$), although there was more variation in the controls.

Trends for haemoglobin were similar in the control and experimental animals, with values oscillating between 50 and 100 g/l, which is within the normal range of variation for ICR mice. Red blood cell counts tended to increase over time, with this increase being significant ($p < 0.05$) only within the control group. RBC values remained within the normal range for both groups. The con-

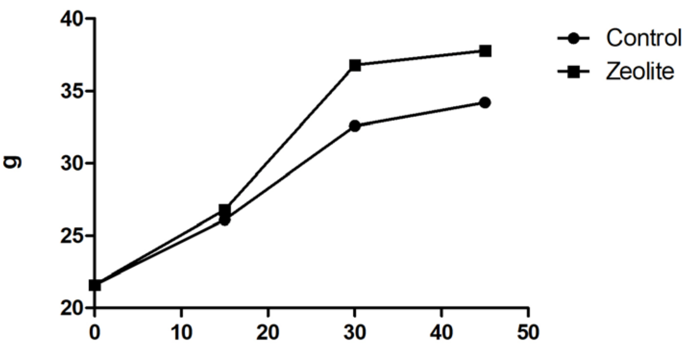

Fig. 1. Growth performance in body mass (g) of control and zeolite-supplemented mice

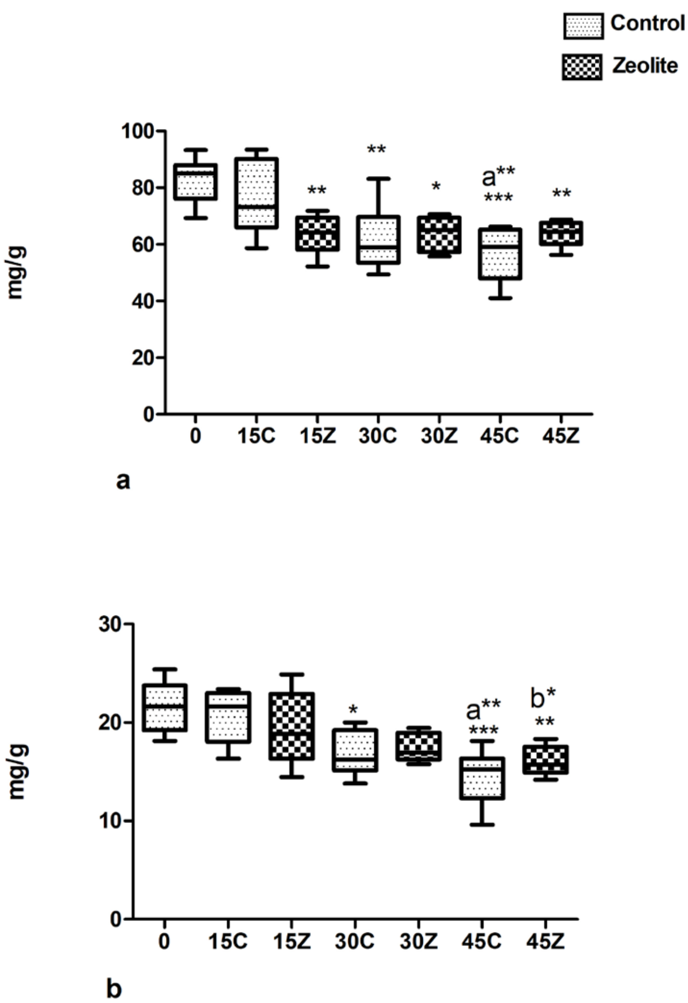

Fig. 2. Liver (a) and kidney (b) indexes, expressed in mg/g live weight, in controls and zeolite-exposed groups. Asterisks indicate significant differences from the control group at day 0 (*p < 0.05; **p < 0.01; ***p < 0.001). The superscript (a) indicates a significant difference from the control group on day 15 and superscript (b) marks a significant difference from the zeolite-exposed group on day 15

Table. 1. Red blood cell, white blood cell and platelet parameters in controls and zeolite-exposed groups. *Legend*: HGB – haemoglobin, HCT – haematocrit, MCV – mean corpuscular volume of erythrocytes, GR – granulocytes, PLT – thrombocytes. Asterisks indicate a significant increase (p <0.05) in the cell counts in the control group between day 45 and day 15. Superscript (a) indicates a significant difference (p <0.05) between the control group at day 45 and the zeolite-exposed group at day 15.

		RBC parameters				**WBC parameters**				**Thrombocytes**
DAY	GROUP	**RBC ($\times10^{12}$/L)**	**HGB (G/L)**	**HCT (L/L)**	**MCV (fL)**	**WBC ($\times10^{9}$/L)**	**LYM (%)**	**MO (%)**	**GR (%)**	**PLT ($\times10^{9}$/L)**
0	**control**	3.32±1.33	69.57±22.07	0.25±0.09	46.83±1.58	4.30±1.67	3.06±1.17	0.26±0.14	2.16±0.82	404.0±219.1
15	**control**	2.68±0.60	54.43±12.88	0.25±0.10	48.71±5.36	3.67±1.46	3.76±0.78	0.10±0.04	1.54±0.41	379.1±153.0
	zeolite	2.41±0.46	45±5.89	0.19±0.07	48.78±6.50	2.23±0.26	2.09±1.18	0.10±0.05	1.06±0.45	285.8±60.2
30	**control**	5.32±2.02	81.14±29.48	0.25±0.09	46.81±1.55	6.02±1.28	3.48±1.36	0.26±0.14	2.15±0.91	432.6±189.5
	zeolite	3.90±1.44	56±25.24	0.19±0.07	48.20±2.96	7.21±2.24	3.83±1.50	0.09±0.01	2.74±0.61	410.7±155.7
45	**control**	$5.88\pm2.59^{*a*}$	82.86±37.26	0.26±0.11	44.04±4.47	$10.1\pm7.43^{*a*}$	5.45±4.11	0.68±0.28*	$3.99\pm2.94^{*a*}$	592.3±305.2
	zeolite	4.17±1.10	61.75±14.38	0.21±0.05	50.35±1.25	7.75±2.13	4.41±1.40	0.09±0.01	2.69±0.75	418.5±71.5

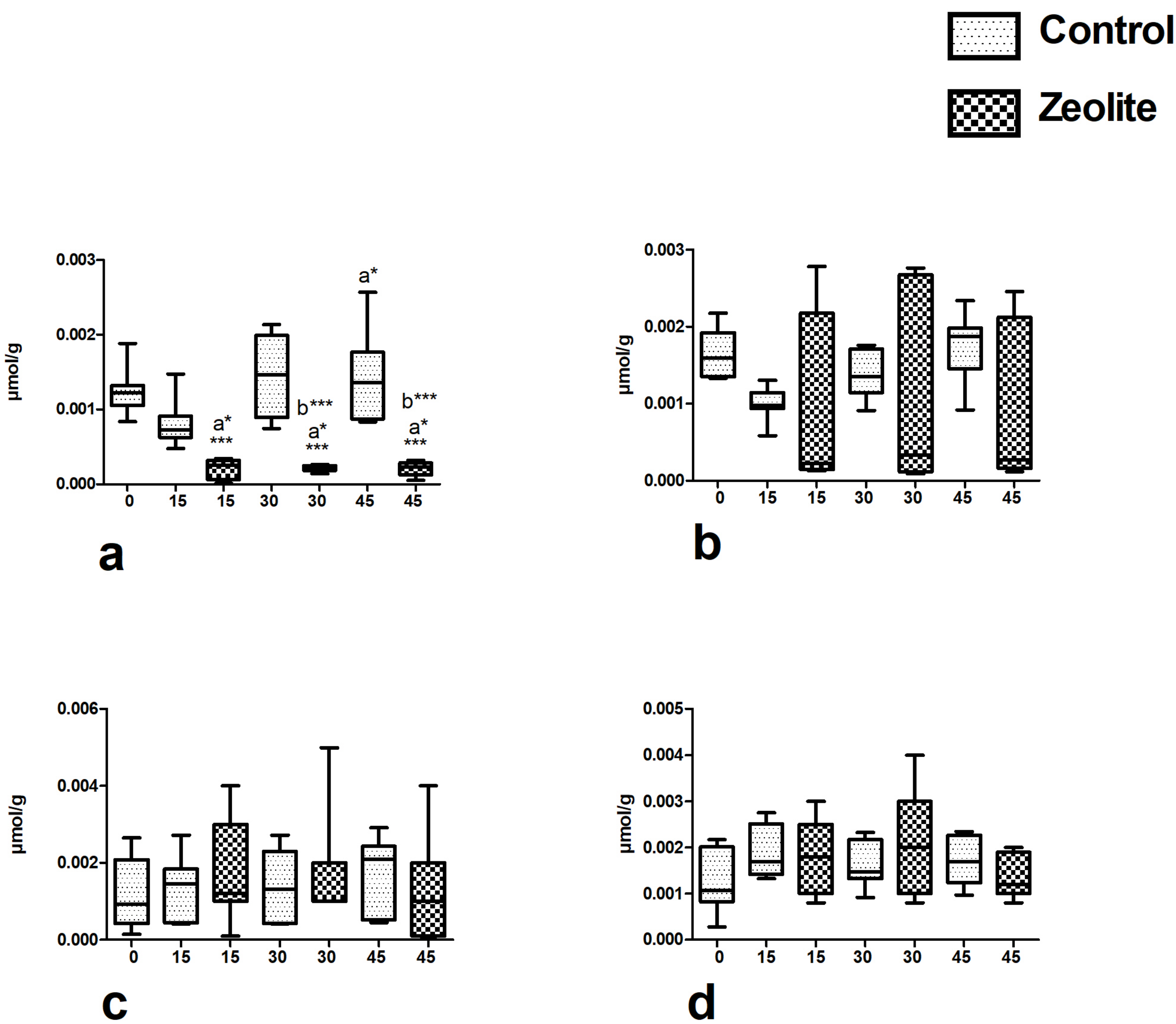

Fig. 3. MDA and total glutathione in the liver and kidneys of control and zeolite-exposed mice: a) total glutathione in the liver; b) total glutathione in the kidney; c) MDA in the liver; d) MDA in the kidney. *** statistically significant difference ($p < 0.001$) with day 0; superscript (a*) indicates significant differences ($p < 0.05$) with the control group at day 15; superscript (b***) marks significant differences ($p < 0.001$) between the zeolite-exposed group and control group.

trol group exhibited a slight increase in total WBC and LYM at day 45, in contrast to the MO count, which demonstrated a statistically significant rise at day 45. Zeolite-supplemented mice, however, had a less pronounced increase. No significant differences were observed between the groups concerning WBC differential counts. Platelets had average values ranging from 300 to 500 G/L, while granulocyte counts ranged from 1.500 to 5.500 cells per μL of blood. Moreover, % granulocytes and % monocytes were virtually identical between the two groups. Thrombopoiesis was within the normal range for both groups with PLT (250-550 x 10^9/L) and MPV (7.71±1.94 fL) parameters falling within the reference range.

Oxidative stress determination

Oxidative stress measurements did not indicate significant differences between controls and zeolite-exposed groups, exempt a significant ($p <0.001$) decrease of glutathione levels in the liver of zeolite-exposed mice. (Fig. 3). The levels of MDA in the liver and kidney showed similar dynamic patterns in both groups. The relationship of MDA Levels with glutathione (Fig. 4) shows that there is an inverse linear relationship between levels of MDA and glutathione, which means that the greater the MDA level, the lower the level of glutathione in the body.

Neither MDA parameter nor total glutathione attained values indicating potential liver or kidney oxidative stress damage.

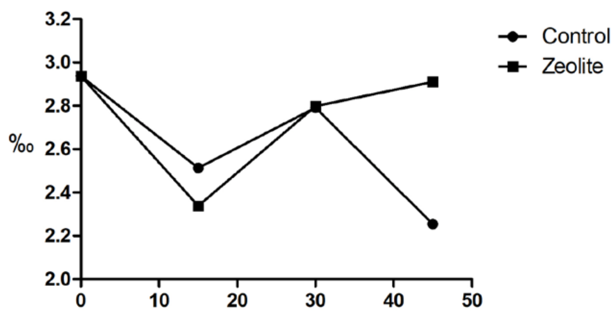

Fig. 4. Average micronucleus frequency (‰) in control and zeolite-exposed mice during the experiment trial.

In vivo micronucleus test

No statistically significant differences (ANOVA, $p > 0.05$) were observed in the formation of MNs among peripheral erythrocytes between the control and the zeolite-supplemented groups. Throughout the experiment, all groups remained below 5 MNs/1000 erythrocytes, which is the established reference limit for laboratory mice. (Fig. 4).

Discussion

Zeolites are increasingly recognized as an efficient strategy to combat the toxic burden of environmental pollution (Collela 2011, Dolanc et al. 2023). Many studies in the literature demonstrate that the administration of zeolite to animals or humans, even for several months, does not show any evidence of harmful reactions or adverse changes to their biochemical and haematological profiles (Martin-Kleiner et al. 2001, Alexopoulos et al. 2007). One of the first comprehensive assessments of subchronic toxicology of tribomechanically-micronized clinoptilolite *in vivo* was performed by Pavelić et al. (2001b). The results are in agreement with those obtained in the present study (Ozesmi et al. 1985, Temel & Gundogdu 1996, Adamis et al. 2000). Consequently, natural clinoptilolites have been approved in several countries' pharmacopeias and authorized for specific drug production and marketing purposes (Collela 2011).

The current results indicate that purified and sodium-exchanged natural clinoptilolite from the "Beli plast" deposit shows a dominance of nontoxic elements and ions in the chemical composition of the sorbent. It is expected that, in the course of its passage through the gastrointestinal tract of the animals, the modified zeolite might release small amounts of Na^+, K^+, Ca^{2+}, or very small amounts of Mg^{2+} ions, all of which are physiologically available, minor biogenic elements and non-toxic in their ionic form.

The observed growth performance and increase in weight gain under clinoptilolite supplementation are unexplained. Nevertheless, similar results have been reported by other authors for pigs (Prvulović et al. 2007), Turkey poults (Hcini et al. 2018), broilers (Qu et al. 2019) and lambs (Tánori-Lozano et al. 2022). The values of organ indices and oxidative stress measurements indicate that there are not any significant deviations in the measured parameters between controls and zeolite-exposed groups. Pavelic (2018) discusses the ability of clinoptilolite to reduce oxidative stress levels in intoxication or tumor findings. The present study proves that the mineral itself does not induce it. Several studies have indicated that adding clinoptilolite to animal feed can result in lower levels of oxidative stress (Hcini et al. 2018, Qu et al. 2019). MDA is a compound that can describe the activity of free radicals in cells so it is used as one of the indications of oxidative stress caused by free radicals. Stress factor deficiency did not result in a change in MDA levels and a corresponding increase in glutathione levels. This suggests that the administration of clinoptilolite reduces oxidative activity and enhances the antioxidant response as revealed by Saribeyoglu et al. (2011). The exact mechanisms of zeolites 'antioxidant effects are not well known. In effect, zeolite is not absorbed into the blood from the gut. The effects of zeolite may be due to an indirect interaction with biochemical systems, removal of waste and toxins from the gut, improvement of the immune system through the mucosal-related intestinal lymphoid tissue and an increase in the bioavailability of minerals that are important co-factors for some enzymes (Dogliotti et al. 2012, Pavelic et al. 2001, Ivkovic et al. 2004)

Haematological parameters, including erythropoiesis (RBC, HGB, HCT, MCV,), thrombopoiesis (PLT,) and differential WBC counts (Total WBC, GR) fall within the expected reference value range for ICR mice (Serfilippi et al. 2003, Shin et al. 2017). In a study performed by Martin-Kleiner et al. (2001), effects of clinoptilolite on serum chemistry and haematopoiesis were evaluated in mice. The authors showed that the dietary intake of clinoptilolite is well tolerated and confirmed by stable body mass and absence of changes in hematopoiesis in clinoptilolite-supplemented mice. Given the data from the micronucleus test, there is no evidence of exogenous gene toxicity in control and zeolite-supplemented mice. This is in accordance with an earlier study, which used chromosomal aberrations as an endpoint. They did not find statistically significant differences ($p>0.1$) in the mitotic indices of the control mice so as in healthy mice supplemented with clinoptilolite (Topashka-Ancheva et al. 2012).

Conclusions

Clinoptilolite remains in the animal's gastrointestinal tract and exhibits chemical stability in low pH conditions, such as in the stomach of mice (pH = 2.5–3.0). It does not degrade within the tract and poses no metabolically activated toxicity. Clinoptilolite's structural molecules are inert and only a few trace metal ions can escape from the crystals. Although we did not observe a reduction in TBARS values, it is conceivable that other experimental models show lower levels of oxidative stress. The present study shows that clinoptilolite from the "Beli plast" deposit in Bulgaria, after appropriate mechanical purification and sodium exchange, is safe and suitable as a feed supplement for animals, both for heavy metal detoxification and as a growth-stimulating additive. No toxicity was observed at the tested concentration of 12.5 wt% of the total feed volume. Due to the lack of any acute toxicity at the applied doses of the used zeolite, it was impossible to determine the LD50 of the sorbent and was assumed practically non-toxic. It is important to note that an increase in sorbent concentration would worsen the nutritional quality of the animal food.

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