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Protective effect of modified natural clinoptilolite against lead-induced genotoxicity in laboratory mice

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Abstract. Lead (Pb) is a potent toxic and genotoxic metal that accumulates in mammalian tissues, induces oxidative stress, and disrupts genomic integrity. Creating effective strategies to reduce Pb bioavailability and lessen its harmful effects remains a key priority in toxicology. This study examined the impact of 15, 30, and 45 days of sub-chronic Pb exposure on tissue accumulation, fecal elimination, and genotoxicity in ICR (CD-1) mice, as well as the potential protective role of a specifically activated, Na-modified natural clinoptilolite from the "Beli Plast" deposit (Eastern Rhodopes, Bulgaria). Three experimental groups were established: a control group, a Pb-exposed group (PbW) receiving 0.00125 M $Pb(NO_3)_2$, and a Pb-exposed+clinoptilolite (PbW+Z) group fed a diet containing 12.5 wt.% clinoptilolite. ICP-MS was used to measure Pb levels in liver, kidney, and feces, and genotoxic effects were assessed using an in vivo micronucleus assay in peripheral erythrocytes. Pb exposure also induced a time-dependent increase in micronucleus frequency, reflecting cumulative genotoxic damage. Micronucleus frequency was markedly elevated in Pb-exposed mice but significantly reduced at all time points when clinoptilolite was present, suggesting protection against Pb-induced genomic instability. Overall, zeolite effectively reduced the total burden of Pb in organisms, increased fecal elimination, and reduced Pb-induced genotoxicity. These findings emphasize zeolite as a promising low-cost protective sorbent for reducing heavy metal toxicity and preventing long-term biological damage for organisms chronically exposed to lead.

Key words: clinoptilolite, lead, mice, genotoxicity, micronuclei in erythrocytes.

Introduction

Lead (Pb) toxicity in mammals is well established, with numerous studies in small rodents showing effects on multiple organs, including nephrotoxicity, hepatotoxicity, hematological changes, and disruption of cellular homeostasis. Chronic and acute Pb exposure increases oxidative stress, disrupts mitotic processes, and causes systemic DNA damage in organisms, with effects that vary depending on the organ and exposure pattern (García-Lestón et al., 2010; Martinez-Morata et al., 2023). Evidence indicates that the liver and kidneys are highly exposed and metabolically active organs, making them common targets for chemical toxicity (Topashka-Ancheva et al., 2012). Repeated exposure to genotoxic compounds frequently leads to organ-specific damage alongside measurable systemic effects, including DNA damage (Beltcheva et al., 2022). Oral Pb doses in mice were associated with DNA damage, increased mitochondrial reactive oxygen species (ROS), and lipid peroxidation (MDA) (Fan et al., 2020).

Given the significant health risks associated with Pb exposure, developing effective strategies to reduce its bioavailability and mitigate its genotoxic effects is a major scientific priority. Zeolites, particularly clinoptilolite, are widely used for the removal of heavy metals from various solutions

and have well-established applications in animal nutrition and health (Pond et al., 1995; Mumpton, 1999; Bish & Boak, 2001). Their effectiveness derives from their high cation-exchange capacity, which enables interactions with ions such as NH_4^+, Pb^{2+}, Cd^{2+}, Cu^{2+}, Cs^+, and others in animal organisms (Pavelić & Hadžija, 2003; Rhodes, 2007; Korkuna et al., 2008). The highly porous structure, large surface area, and substantial ion-exchange capacity of the clinoptilolites make them particularly effective in adsorbing heavy metals and limiting their bio-availability. Mechanical activation, or micronization, increases surface area and accelerates Pb ad-sorption in vitro, thereby improving uptake kine-tics (Valverde et al., 2002).

The reduction in Pb bioaccumulation is attributed mainly to clinoptilolite's ability to bind Pb^{2+} ions in the gastrointestinal tract, thereby limiting their absorption into tissues (Kraljević Pavelić et al., 2018). Controlled rodent studies after 2000 indicate that modified natural clinoptilolite, administered orally, reduces Pb^{2+} absorption and tissue burden and attenuates Pb-associated chromosomal and oxidative DNA damage via gut sequestration and indirect antioxidant effects (Kraljević Pavelić et al., 2018; Beltcheva et al., 2022; Aleksieva et al., 2025; Beltcheva et al., 2024, 2025). However, most studies have focused on general toxicity and metal clearance, while information about their specific effects on Pb-induced genotoxicity, especially for clinoptilolite from Bulgarian deposits, remains limited. Bulgaria contains substantial deposits of high-quality zeolites, primarily clinoptilolite, in the Eastern Rhodope Mountains.

The frequency of micronuclei (MNs) in mammalian erythrocytes serves as a sensitive and rapid indicator of genotoxicity, reflecting irreversible genomic damage, and has been successfully employed as a genotoxic biomarker in rodent studies (Mitkovska et al., 2012, 2020). Prolonged exposure to elevated concentrations of Pb exerts genotoxic effects on peripheral blood and bone marrow cells in rodents, including DNA damage, leading to mutations, chromosomal aberrations, and the formation of MNs (Chassovnikarova et al., 2025).

The present study aimed to conduct targeted ecotoxicological experiments to evaluate the ability of Bulgarian clinoptilolite to adsorb Pb in zeolite-supplemented ICR mice and reduce genotoxic damage. The hypothesis proposed that administering zeolite would effectively lower Pb accumulation in target organs and diminish associated genotoxic effects, highlighting its potential as a protective agent against Pb-induced genotoxicity.

Materials and methods

Clinoptilolite

The modified clinoptilolite from the "Beli Plast" tuff deposit in Bulgaria was ground, sieved, and the <0.15 mm fraction was Na-exchanged by stirring 100 g with 1000 ml of 1 M $NaNO_3$ at 80°C and 500 rpm in a closed vessel. It was then washed with deionized water, dried at 60°C, and the mineral composition was analyzed using Powder X-ray Diffraction. Details are described in Beltcheva et al. (2025).

Animals and experimental design

The investigation employs a three-group experimental design using ICR (CD-1) mice. All animals are of the same sex and age, weighing between 18.0 and 22.0 grams at the start of each experiment. The animals are bred in a vivarium and housed in individually ventilated cages that meet European size standards - bedding material is supplied by an ISO 2000–accredited producer. Mice are acclimated for 7 days before the experiment. During the study, environmental conditions are maintained at 19–23°C and 45–60% humidity, with a 12-hour light/dark cycle. The animals receive pelleted food and have free access to both food and water, which, along with bedding, are inspected daily and replaced as needed. No animals are medicated or vaccinated.

The three experimental groups were as follows: a control group (C), which received a standard rodent diet and water ad libitum; a Pb-exposed group (PbW), which received an oral solution of 0.00125 M $Pb(NO_3)_2$ in water along with a standard rodent diet; and a Pb plus clinoptilolite group (PBW+Z), which was administered the same 0.00125 M $Pb(NO_3)_2$ solution orally and provided a standard rodent diet supplemented with 12.5 wt.% powdered modified natural clinoptilolite. Each experiment lasted 45 days, during which blood samples were collected from the tested animals on days 0, 15, 30, and 45.

All experiments were conducted in accordance with Ordinance No. 20/01.11.2013 on the protection and welfare of animals, the Animal

Protection Act of 31 January 2008 of the Republic of Bulgaria, and were approved by the Institute of Biodiversity and Ecosystem Research Ethical Committee.

Lead bioaccumulation

Pb bioaccumulation in the liver and kidneys was quantified using a Perkin Elmer SCIEX DRCe ICP-MS system equipped with a cross-flow nebulizer. A detailed description of the analytical methodology is provided in Beltcheva et al. (2025).

Micronucleus test

Genotoxicity at environmentally relevant $Pb(NO_3)_2$ concentrations was assessed via an in vivo MN test using a modified acridine orange method, in which PBS was replaced with Sörensen's phosphate buffer (pH 7.4) (Mitkovska et al., 2012). Imaging was performed on an OPTIKA B-383 FL fluorescence microscope (400×, blue filter: 460–490/515 nm). MNs were defined by typical yellow-green fluorescence, round/oval morphology, and a size ≤ one-third of the main nucleus. Micronucleus frequency was calculated per 2000 erythrocytes (‰).

Statistical analyses

To evaluate data normality, the D'Agostino–Pearson test was used. A two-way ANOVA was performed separately for each tissue and feces to examine the effects of treatment, time, their interaction, and to estimate MN frequency in peripheral erythrocytes. These analyses were conducted using Prism software, version 9.0 (GraphPad Software, San Diego, CA, USA), with a significance level set at $p \leq 0.05$.

Results

Pb bioaccumulation

Pb concentrations in the kidney, liver, and feces were evaluated at days 15, 30, and 45 in mice exposed either to Pb alone (PbW) or to Pb supplemented with zeolite (PbW+Z). Two-way ANOVA demonstrated significant effects of treatment, time, and their interaction in all three tissues. In the kidneys, Pb levels in the PbW group rose from 142.7 ± 78.8 mg/kg on day 15 to a peak of 360.7 ± 159.9 mg/kg on day 45 (Fig. 1a). In contrast, the PbW+Z group maintained significantly lower concentrations (82.5 ± 47.5 mg/kg to 92.1 ± 43.2 mg/kg). Both treatment ($p < 0.0001$) and time ($p < 0.0001$) significantly affected Pb accumulation, and a strong treatment × time interaction ($p < 0.0001$) was observed, indicating that the impact of zeolite changed across the exposure period. Treatment accounted for the largest proportion of variance (36.4%), suggesting that zeolite markedly reduced renal Pb levels, particularly at later time points. The interaction accounted for an additional 25% of the variance, aligning with the increasingly large gap between the PbW and PbW+Z curves, especially at day 45.

A similar trend was observed in the liver (Fig. 1b), where Pb levels in the PbW group increased from 70.6 ± 42.6 mg/kg to 178.1 ± 86.9 mg/kg or remained elevated over time, whereas mice supplemented with zeolite displayed consistently lower hepatic Pb concentrations across all time points (12.6–23.8 mg/kg). Two-way ANOVA showed significant effects of treatment ($p < 0.0001$), time ($p < 0.0001$), and their interaction ($p < 0.0001$). In the liver, treatment effects were even more significant ($p < 0.0001$), accounting for nearly 40% of the total variance and representing the strongest treatment effect among all tissues, demonstrating that zeolite effectively suppresses hepatic Pb accumulation. The significant interaction (20.1% of total variation) indicates that temporal changes in liver Pb concentration differed significantly between the two groups.

In contrast, in fecal samples, the PbW group showed a moderate increase in Pb levels over time, reaching 167.22±147.45 mg/kg on day 15 and peaking at 296.05±199.58 mg/kg on day 30 (Fig. 1c). In contrast, the PbW+Z group exhibited markedly elevated fecal Pb concentrations, especially on days 15 (417.8±258.34 mg/kg) and 30 (456.8 ± 278.56 mg/kg), followed by a slight decline on day 45. This pattern suggests enhanced gastrointestinal elimination of Pb in the presence of zeolite. Two-way ANOVA confirmed significant effects of supplementation ($p < 0.0001$), time ($p < 0.0001$), and their interaction ($p = 0.0003$). Treatment accounted for 31.1% of the variation, time for 19.4%, and the interaction for 12.8%. The significant interaction indicates that the pattern of Pb excretion differed between groups, with an apparent early rise in fecal elimination in zeolite-treated mice.

Across all tissues, Pb concentration changed significantly over time and differed markedly between treatments. Overall, zeolite supplementation significantly decreased Pb levels in the kidney

and liver, while enhancing fecal Pb excretion. Pb bioaccumulation was reduced, with a twofold decrease in the kidneys by day 15 and a tenfold decrease in the liver by day 30 among the experimental groups. Meanwhile, clinoptilolite's capacity to absorb Pb ions increased Pb gastrointestinal elimination via feces, which was more than twice as high, particularly during the early and mid-phases of exposure. The presence of a significant treatment × time interaction in the kidney, liver, and feces indicates that zeolite not only reduces Pb accumulation in organs but also alters the timing of Pb distribution and elimination.

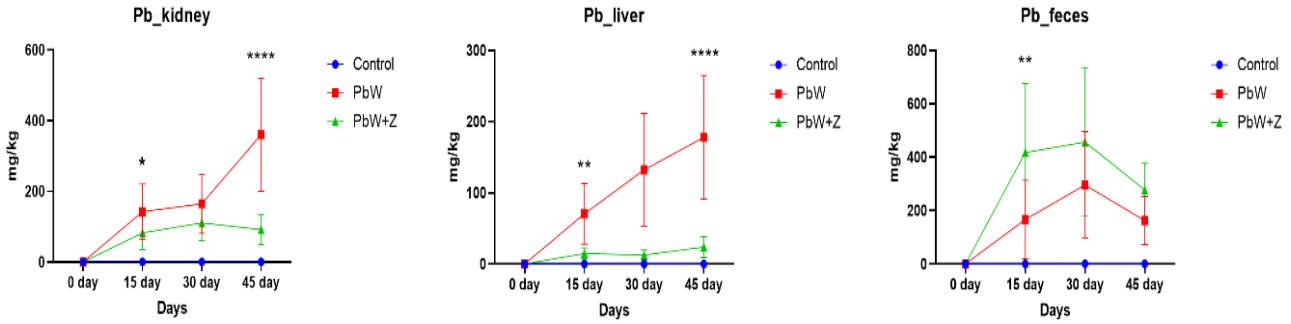

Fig. 1. Lead bioaccumulation (mg/kg) in the kidneys, liver, and feces of mice exposed to Pb (PbW) or to Pb supplemented with zeolite (PbW+Z) on days 15, 30, and 45. Asterisks indicate significant differences at * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ for comparisons between treatments within the same time point.

Micronucleus frequency in peripheral erythrocytes

The frequency of micronucleated erythrocytes was evaluated at days 15, 30, and 45 to assess the genotoxic effects of Pb exposure and the potential protective role of zeolite (Fig. 2). Two-way ANOVA showed significant main effects of treatment ($p < 0.0001$) and time ($p < 0.0001$), as well as a highly significant interaction between treatment and time ($p < 0.0001$). Treatment accounted for 35.65% of the total variation, indicating a substantial impact of Pb and zeolite on MN formation. Time contributed 24.73%, reflecting the progressive accumulation of genotoxic damage during prolonged Pb exposure. The interaction accounted for 26.34% of the variation, indicating that the induction period of MNs varied significantly between groups.

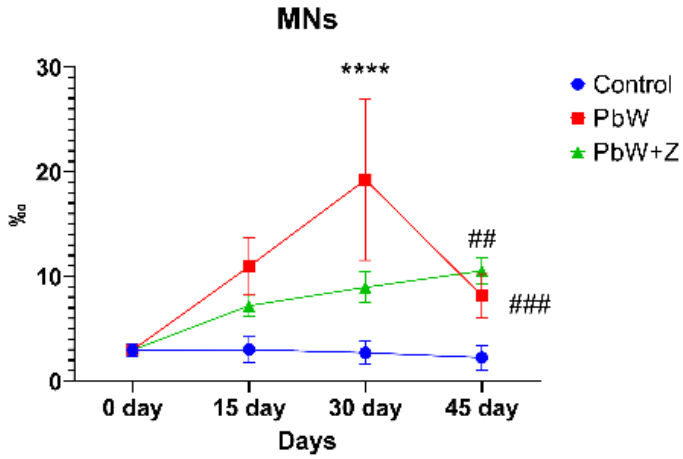

Fig. 2. The frequency of micronuclei (MN/2000) in the peripheral erythrocytes of the mice from the control group (C), lead (PbW), and lead supplemented with zeolite group (PbW+Z) at days 15, 30, and 45. Asterisks indicate significant differences at **** $p<0.0001$ for comparisons between treatments within the same time point.

The mice in the PbW group showed a significant increase in micronucleus frequency over time, while the PbW+Z group consistently exhibited significantly lower levels at all time points. These findings suggest that zeolite effectively reduces Pb-induced genotoxicity and influences its progression over time.

Discussion

Pb accumulation and zeolite effects

The present study confirms and further substantiates the previously observed effects of clinoptilolite administration on Pb concentration profiles during subchronic intoxication in small rodents (Beltcheva et al., 2012, 2015; Topashka-Ancheva et al., 2012; Kraljević Pavelić et al., 2018). The focus of the current investigation was the evaluation of Pb-induced genotoxicity, assessed through the MN frequency in peripheral erythrocytes. Pb exhibits a pronounced capacity for bioaccumulation in mammalian organisms, with progressive retention in the liver and kidneys (Fang et al., 2023), resulting in marked disruptions of metabolic, detoxification, and renal filtration processes. Chronic lead accumulation further elicits a spectrum of pathological alterations - including heaptic steatosis and inflammation, renal tubular degeneration, hematological dysfunction, and systemic oxidative and genotoxic stress - which collectively promote chromosomal breakage and mitotic errors, ultimately manifested as elevated MN formation in peripheral erythrocytes (Stopper & Müller, 1997; Jagetia & Aruna, 1998; Jankeer & El-Nouri, 2009; García-Lestón et al., 2010; Takano et al., 2015; Andjelkovic et al., 2019).

Previous studies have shown that Bulgarian clinoptilolites from deposits in the Eastern Rhodopes (Beltcheva et al., 2012, 2015; Topashka-Ancheva et al., 2012) help maintain the normal physiological status of organisms during chronic heavy-metal intoxication by adsorbing substantial amounts of Pb^{2+}. Evidence from multiple authors further indicates that the Na-enriched form of modified clinoptilolite exhibits the highest static ion-exchange capacity not only for Pb^{2+} but for Cd^{2+}, NH_4^+, and other cations (Rhodes et al., 2007; Haemmerle et al., 2021; Klaassen et al., 1999). Consequently, the modification applied in the present study can be considered effective for detoxification purposes. The analysis and comparison of the results obtained in the present study regarding Pb bioaccumulation in the target organs of mice, before and after zeolite supplementation in their diet, are even more encouraging than those observed with the KLS-10-MA modification (Beltcheva et al., 2015). While the KLS-10-MA modification resulted in a reduction of Pb bioaccumulation in the liver by up to sevenfold, the Na-modified form achieved an almost tenfold decrease in the liver on day 30 of the experiments. The present study demonstrates that supplementation with the Na-enriched form of modified clinoptilolite from the Beli plast deposit has a more pronounced protective effect against Pb accumulation in ICR mice, with consistent reductions in renal and hepatic Pb levels and enhanced fecal elimination. The two-way ANOVA revealed strong treatment effects across all tissues, indicating that zeolite substantially alters Pb biodistribution.

Notably, the interaction between treatment and time was significant in all tissues, indicating that the magnitude of the zeolite effect is not constant but evolves over the exposure period. In the kidney, which is a primary target organ for Pb toxicity, zeolite markedly suppressed Pb accumulation, especially at later stages of exposure. This suggests that zeolite may interfere with chronic Pb retention mechanisms, possibly by binding Pb in the gastrointestinal tract and reducing systemic absorption (Beltcheva et al., 2015; Dolanc et al., 2023). The liver showed the most potent treatment effect of all tissues, indicating that the Na-enriched form of modified clinoptilolite from the Beli plast particularly limits hepatic Pb burden. Since the liver plays a central role in detoxification and metal redistribution (Alamri, 2018), this reduction is likely physiologically meaningful and may mitigate downstream toxic effects. The increased Pb concentrations in the feces of zeolite-treated animals strongly support the hypothesis that zeolite enhances gastrointestinal elimination of Pb (Mercurio et al., 2016; Khalilzadeh et al., 2025; Teimouri et al., 2025). This mechanism is consistent with the known cation-exchange capacity and adsorption properties of natural zeolites, which can bind heavy metals in the digestive tract and prevent their reabsorption (Beltcheva et al., 2012, 2015; Senila & Cadar, 2024). Together, these findings indicate that the Na-enriched form of modified clinoptilolite from the Beli plast not only reduces Pb uptake but also modifies its temporal kinetics in the body. The significant treatment × time inter-

actions suggest that zeolite becomes increasingly effective with prolonged exposure, likely due to cumulative Pb binding and sustained promotion of fecal excretion. Overall, the data support the potential of zeolite as a low-cost, safe, and effective agent for reducing Pb body burden and mitigating organ toxicity.

Pb genotoxic effect and zeolite impact

The MN results provide strong evidence that Pb causes significant genotoxic damage in peripheral erythrocytes, consistent with its known induction of chromosome instability, genomic rearrangements, and mutagenesis (Ye et al., 2019; Nagaraju et al., 2022). The progressive rise in MN frequency in the PbW group reflects cumulative DNA damage during chronic exposure. This aligns with the known genotoxic potential of Pb to significantly induce MN formation, a key biomarker of chromosomal damage, in various in vivo models (Hemmaphan & Bordeerat, 2022). This genotoxicity is attributed mainly to Pb^{2+} acting as an aneugenic agent, disrupting the microtubule network and leading to whole chromosome loss, though clastogenic (chromosome breakage) effects are also implicated (Bonacker et al., 2005; Krupina et al., 2021).

Notably, the Na-activated natural clinoptilolite significantly lowered MN formation at all time points, indicating that it not only reduces systemic Pb burden but also alleviates Pb-induced genetic instability. This is consistent with previous research on ICR (CD-1) mice given the same Na-modified clinoptilolite plus cadmium, which did not increase MN frequencies and was linked to reduced cadmium-induced DNA damage in an in vivo MN assay, demonstrating a protective effect on erythroid/genotoxicity endpoints (Beltcheva et al., 2025). A natural-zeolite-based nanohybrid (eugenol@natural zeolite) showed lower genotoxicity and fewer micronuclei compared with an unencapsulated clove powder comparator in the reported assays (da Silva et al., 2025). Another study reported that modified natural clinoptilolite KLS-10-MA from Bulgarian deposits, when used as a Pb adsorber in mice, was both safe and effective, without causing chromosomal damage (Topashka-Ancheva et al., 2012).

The significant treatment × time interaction further indicates that zeolite modifies the temporal kinetics of genotoxicity, preventing the escalation of DNA damage typically observed with prolonged Pb exposure. This pattern closely parallels the biochemical findings, where zeolite reduced Pb accumulation in the kidney and liver while increasing fecal elimination. Together, these data suggest that decreased systemic Pb levels - due to reduced absorption and enhanced excretion – translate directly into lower genotoxic stress. Thus, zeolite exerts a dual protective role by limiting Pb biodistribution and preserving genomic integrity. Our results demonstrate that sub-chronic Pb exposure induces MN formation in peripheral erythrocytes and underscore the efficacy of the Na-modified natural clinoptilolite from the Beli plast deposit as an in vivo Pb^{2+} sorbent with pronounced genoprotective effects.

Conclusions

This study demonstrates that zeolite provides substantial protection against Pb toxicity by reducing tissue accumulation, enhancing fecal elimination, and preventing genotoxic damage. Sub-chronic Pb exposure resulted in progressive accumulation of Pb in the kidney and liver, accompanied by a marked increase in micronucleus frequency in peripheral erythrocytes. Zeolite supplementation significantly changed this: Pb concentrations in organs were consistently lower, fecal excretion was markedly increased, and micronucleus formation was strongly suppressed. These results indicate that zeolite acts by limiting Pb absorption, promoting its elimination, and reducing systemic and genetic toxicity. The toxicological and cytogenetic findings provide evidence that zeolite is an effective protective agent against Pb exposure. Its low cost, safety, and efficacy support its potential for mitigating heavy metal toxicity in environmental and public health settings.

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