Evaluation of the Cytotoxic Effects of heavy metals in laboratory animals.

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Abstract:

Scientific evidence indicates that exposure to heavy metals commonly present in consumer products, water, and air can lead to significant cytotoxic and genotoxic effects. This study aimed to evaluate the combined effects of zinc (Zn) and cadmium (Cd) exposure on the genetic makeup of white laboratory mice (C57BL). Heavy metals, particularly cadmium, have been shown to induce DNA damage in both humans and animals. A total of 150 mice were divided into three equal groups. The first group received drinking water containing cadmium salts at a concentration of 0.685 mg/L, the second group received zinc salts at 0.567 mg/L, and the third served as a control group. The exposure period lasted 90 days. Cytotoxic and genotoxic effects were assessed using the comet assay, mitotic index (MI), blast index (BI), and micronucleus (MN) test in lymphocytes. The results demonstrated that chronic exposure to cadmium caused a significant increase in the mitotic index (37.67 ± 4.95) and micronucleus frequency (1.42 ± 0.26) at a significance level of ($P < 0.01$), with the highest values observed in the third month. In contrast, no significant differences were observed between the zinc-exposed group and the control group. These findings confirm the genotoxic potential of cadmium by demonstrating its ability to induce notable cytogenetic alterations. Accordingly, continuous monitoring of environmental exposure to heavy metals remains essential for assessing potential risks to the health and genetic integrity of living organisms.

Key words: Micronucleus, mitotic index, blast index, cadmium, lymphocyte.

1. Introduction

The health effects of heavy metals are contagious. High levels of heavy metals are present in the bodies of 65% of North Americans. Heavy metal exposure frequently results in neurological conditions including Parkinson's disease, skin disorders, cardiovascular disorders, carcinoma, tumors, uncommon immunological disorders, and degenerative diseases [1]. Reactive oxygen

species are produced by heavy metals, which also alter DNA repair mechanisms. Our health is severely harmed by these heavy metals. Even at very low concentrations, heavy metals can be toxic or harmful to the body depending on their chemical makeup (2). In addition to creating a source of DNA damage through the production of reactive oxygen species, exposure to heavy metals in the environment also causes cellular alterations that may impact the competitive balance between repair mechanisms, changing the development of the DNA repair process. When evaluating exposures that raise the probability of disease starting, it is important to take into consideration the risk of environmental disturbances affecting the DNA repair balance in favor of "error-prone" outcomes. These heavy metals would contribute to the spread of altered DNA and potentially a higher incidence of cancer by encouraging error-prone DNA repair [3]. Reactive oxygen species production, DNA repair inhibition, glutathione decrease, and possibly apoptosis suppression are the primary mechanisms of heavy metal genotoxicity, mutagenicity, and carcinogenicity [4]. Heavy metals can have toxic and even lethal consequences because they can evade regulatory mechanisms such transport, compartmentalization, homeostasis, and binding to specific cell constituents. Given that heavy metals displace vital metals from their proper locations, they can cause cellular functions to malfunction. The primary source of oxidative degradation of biological macromolecules has been identified as metal binding to nuclear proteins and DNA [5].

1.1 Mechanism of DNA damage by certain heavy metals DNA : It suggested that the interaction between the structure and pattern of DNA was the cause of the genetic material's breakdown. This breakdown of the genetic material is mostly caused by genotoxic chemical compounds. Recent research indicates that proteins involved in DNA repair processes, particularly in mismatch and excision repair, are vulnerable to cadmium toxicity. Cadmium's interference and inhibition of DNA repair processes may contribute to a higher risk of human tumor development [6].The IARC has designated cadmium as a human carcinogen because it is a hazardous or non-essential metal that can damage DNA even at low concentrations [7].

1.2 Heavy metal resources in soils: Minerals and metalloids with densities higher than > 5 g cm-3 will be referred to as heavy metals. Soil may naturally contain heavy metals [8] or may be contaminated by human interference. The transfer of volcanic emissions from continental soils and the weathering of mineral-rich rocks as a result of extended air exposure are two examples of natural sources of heavy metals that contribute significantly to the soil [9]. Also, other industrial processes, such as the exploitation of mines and smelters, the use of metal-based pesticides and metal-enriched sewage sludge in agriculture [10], the consumption of fossil fuels, the metallurgical industries, electronics, military training and weapons, etc., can contaminate the soil with natural sources of heavy metals [11]. Metalliferous mining and smelting (such as As, Cd, Cr, Co, Cu, Hg, Ni, Zn), industrial activities (such as As, Cd, Cr, Cu, Pb, Hg, U), atmospheric deposition (such as As, Cd, Cr, Cu, Pb, Hg, U), agriculture (such as As, Cd, Cu, Pb, Se, U, Zn), and waste disposal (such as As, Cd, Cr, Cu, Pb, Hg, Zn). Soil pollution is particularly caused by agricultural activities such the overuse of phosphatic fertilizers for maximum crop yield [12], the careless use of poisonous pesticides, and the excessive use of sewage sludge [13].

1.3 Heavy metal toxicity mechanism: Toxic metal species can bind to proteins, thereby affecting the biological processes of the target molecule. For instance, higher Cu and Zn levels have been observed to significantly impact microbial biomass and soil enzyme activity [14]. The biological function of some proteins with sensitive S groups can be disrupted by toxic metal interactions with thiols and disulphides [15]. Reactive oxygen species (ROS), which are organic byproducts of typical metabolism, are frequently produced by these reactions. Protein folding as well as

apoenzyme binding by cofactors may eventually be affected by the confusion of sensitive thiol groups caused by metal exposure, disrupting the proteins' normal biological activity [16]. Heavy metals can participate in Fenton-type reactions, which are catalytic processes that result in ROS. Through a variety of metabolic pathways, these reactions may put the cell under oxidative stress and greatly raise ROS levels, which may lead to the breakdown of DNA and the destruction of lipids and proteins [16]. Additionally, toxic metals can enter cells through a variety of transporters or pass through the cell membrane and attach themselves to lipophilic carriers. The normal transport of necessary substrates is interfered with by the transporter-mediated uptake of hazardous metals, which leads to competitive inhibition of the transport mechanism. The ATP pool or proton motive force provides energy for this transport mechanism [17]. By removing electrons from the respiratory chain, certain toxic metals might indirectly reduce microbial organisms [18].

1-4 Heavy Metal–Induced Human Disorders: Cadmium is considered one of the most hazardous metals to human health [19], as classified by the International Agency for Research on Cancer (IARC) [20]. Acute exposure to cadmium can lead to inflammation, followed by symptoms such as cough, dryness and irritation of the nose and throat, headache, dizziness, chest pain, pneumonitis, and pulmonary edema [21].

1.5 Carcinogenesis: Continuous exposure to cadmium can cause cancer in humans by changing healthy epithelial cells into cancerous ones that prevent the creation of proteins, DNA, and RNA [22]. Heavy metals prevent xeroderma pigmentosum group A (XPA), which detects DNA degradation, from attaching to DNA. Additionally, cadmium decreases the tumor suppressor p53's ability to attach to DNA [23], which is in charge of base excision repair in DNA exposed to UV light [24]. By preventing DNA repairs at different levels, prolonged exposure to cadmium may cause genomic instability and the formation of tumors [25].

1.6 Infertility: Toxic metal exposure affects the total amount of sperm in semen, an essential part of reproduction [26]. Cadmium exposure can enter testicular cells through voltage-dependent calcium channels and ion transporter chains. Testicular germ cells and sertoli display sperm-head voltage-dependent calcium channeling [27], and exons 7 and/or 8 are eliminated when testicular cadmium levels are elevated [28], which reduces the sperm count [29].

1.7 Cardiovascular abnormality: Maney heavy metals, such as cadmium, can be present in food, tobacco, and the air. In vitro exposure causes endothelial dysfunctions, and in vivo exposure speeds up the creation of atherosclerotic plaque, which thickens the arteries and causes cardiovascular disease [30]. By binding to metallothionein [31], a protein that controls zinc homeostasis and acts as a free radical scavenger [32], cadmium disrupts anti-oxidative stress and increases the production of reactive oxygen species [33].

1.8 Osteotoxicity: A number of heavy metal-induced hormone disruptions, such as those that alter vitamin D metabolic pathways [34], which is associated to cadmium-related bone alterations because calcium is released from bone when parathyroid hormone and calcitonin are not in bloodstream [35]. Cadmium concentrations in estrogen hormone pathways activate the estrogen receptor (ER) and prevent it from attaching to estrogen, which indirectly affects the skeleton and causes osteotoxicity and frequent bone fractures. [36].

1.9 Renal disorder: Itai-itai disease, which is characterized by significantly decreased tubular and glomerular function, is caused on by high-dose cadmium exposure [37]. Long-term low-dose cadmium exposure causes tubular damage, including losses of retinol-binding protein (RBP), glucose, amino acids, phosphate, calcium, β2-MG, and zinc and copper linked to the metal binding protein metallothionein (MT) [38]. Long-term exposure to cadmium causes aberrant

urine with low-molecular-weight proteins, calcium, amino acids, phosphate, and glucose; this is comparable to Fanconi's syndrome, a genetic condition of renal tubular transport that causes kidney damage, with blood cadmium serving as a signal [39].
This study aimed to highlight the importance of continuous environmental monitoring of heavy metals due to their potential risks to the health and genetic integrity of living organisms.

2. Methods and materials:

This experiment was conducted in the laboratories of the Environmental, Water, and Renewable Energy Research and Technology Center, Scientific Research Commission, over a period of 90 days (from April 1, 2019, to July 1, 2019). A total of 100 white mice (male and female) of the C57BL/6 strain, aged 8 weeks and weighing between 35 and 50 g, were used in this study. All animals were maintained under identical experimental conditions, including management, veterinary care, feeding, and housing. The mice were housed in standard cages in an automated, temperature-controlled room (22–25 °C) with a 12-hour light/dark cycle in the animal facility of the Al-Razi Center for Medical and Diagnostic Kits. According to the guidelines of the National Research Council (1995) Committee on Animal Nutrition, animals were provided with a standard diet and had ad libitum access to water. The rats were randomly assigned into three groups, with 50 animals in each group: a control group that received normal drinking water; a second group exposed to cadmium (Cd), in which drinking water was supplemented with cadmium salts at a concentration of 0.0685 g/L (grams per liter) in accordance with Iraqi regulations; and a third group treated with zinc in the form of zinc acetate at a dose of 0.056 g/L.
At the end of the experimental period, blood samples were collected via cardiac puncture. White blood cells were separated, and lymphocytes were isolated from both blood and bone marrow for subsequent analysis. DNA damage was assessed using the comet assay, following the method described by Justus et al. [40].
For sample preparation, lymphocytes were suspended in 1 mL of cold buffer solution and gently mixed for 5 minutes. A volume of 100 µL of the suspension was then mixed with 600 µL of low-melting-point agarose (0.8 g per 100 mL buffer). Subsequently, 100 µL of this mixture was spread onto glass slides pre-coated with agarose. The slides were immersed in a buffer solution containing 0.045 M Tris-borate-EDTA (TBE) at pH 8.4, supplemented with 2.5% sodium dodecyl sulfate (SDS), for 15 minutes. Following this, the slides were placed in an electrophoresis chamber containing TBE buffer (without SDS) and subjected to electrophoresis at 2 V/cm for 2 minutes (100 mA). After electrophoresis, the slides were treated with lysis solution (2.5% SDS) for 15–60 minutes in the dark. DNA staining was performed using acridine orange (15 µL dye in 150 mL buffer). The slides were then examined under cooled conditions (4 °C) using a fluorescence microscope at a wavelength range of 420–490 nm. For each sample, 20 cells were analyzed using an image analysis system based on DNA migration patterns. Quantitative analysis of comet parameters was performed using Image J software.
Statistics: All data presented are the mean values ± SD. Statistical analysis was performed by students t-test to test the significant difference between means at $p<0.01$ level.

3. Results and Discussion:

The results presented in Table (1) demonstrate the effect of cadmium on genetic material in mice blood lymphocytes. The ratio of cell length to cell width reached 1.452 in the cadmium-treated group, compared to 1.164 in the control group, indicating a highly significant difference ($P \leq 0.01$). This increase reflects enhanced DNA damage in lymphocytes exposed to cadmium.

The comet assay revealed variation in the extent of DNA migration among samples, including both control and treated groups. This variation is consistent with previous studies, as different methodologies have been employed by researchers to assess DNA damage using this technique. The comet assay is considered a flexible and sensitive method for detecting DNA strand breaks at the single-cell level, particularly when combined with image analysis techniques (Fig. 1)

Table 1: Differences in metric scale in length-to-width ratios for Cd-group of blood samples

Label	Area	Mean	Angle	Length	L / W ± SE
Sample 1	17	46.318	158.199	15.606	1.452± 0.19
	12	77.578	-120.964	10.75	
Sample 2	12	80.282	-135	11.314	1.34± 0.07
	9	25.984	-20.556	8.433	
Control	8	57.406	-39.806	7.106	1.164± 0.14
	7	65.112	38.66	6.103	

Table 2: Differences in the metric scale in length-to-width ratio for Zn group. of blood samples

Label	Area	Mean	Angle	Length	L / W ± SE
Sample	15	51.895	-36.027	13.744	1.33 ± 0.19
	11	54.166	45	10.371	
Control	16	57.621	-24.775	14.907	1.12 ± 0.07
	14	63.987	53.973	13.333	

One of the simplest approaches for analyzing comet assay results is to determine the proportion of cells exhibiting DNA migration. However, this parameter is strongly influenced by electrophoresis conditions, which can affect the extent of DNA movement and, consequently, the interpretation of results. The micronucleus (MN) frequencies and mitotic index (MI) values are presented in Table (1). Cadmium (Cd) exposure induced a significant increase in MN formation in mice lymphocytes (Figure 1). A statistically significant difference ($P < 0.01$) was observed in both MI and MN frequency in the Cd-treated group with the zinc-treated group and the control group.

Table 3: Cytogenetic parameters including mitotic index, blast index and micronuclei

Test / Group	MI M ± SD	BI M ± SD	Mn M ± SD
Cd- group	37.67 ± 4.95**	45.72 ± 2.73	1.42 ± 0.26**
Zn- group	33.08 ± 3.68	45.21 ± 1.15	0.62 ± 0.09
Control	11.63 ± 2.99	44. 69 ± 2.42	0.49 ± 0.11

** p<0.01

In contrast, no significant differences were observed in the blast index (BI) frequency among mice exposed to cadmium when compared with the zinc-treated and control groups. Cadmium (Cd) exposure resulted in an increase in the mitotic index (MI) in mice lymphocytes. At the tested concentration, Cd induced a dose-dependent increase in MI, showing a statistically significant difference between the treated and control groups ($P < 0.01$). In contrast, no significant change was observed in the blast index (BI).

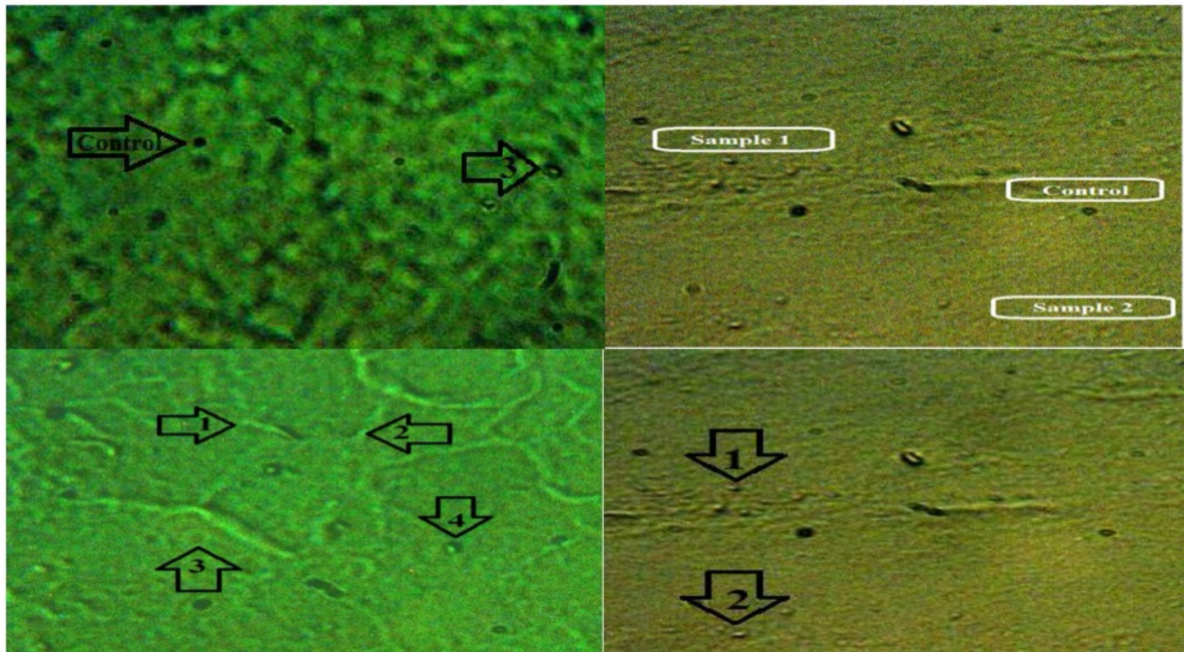

Fig. 1: The damage of genetic material in mice blood lymphocytes which treated with cadmium and zinc.

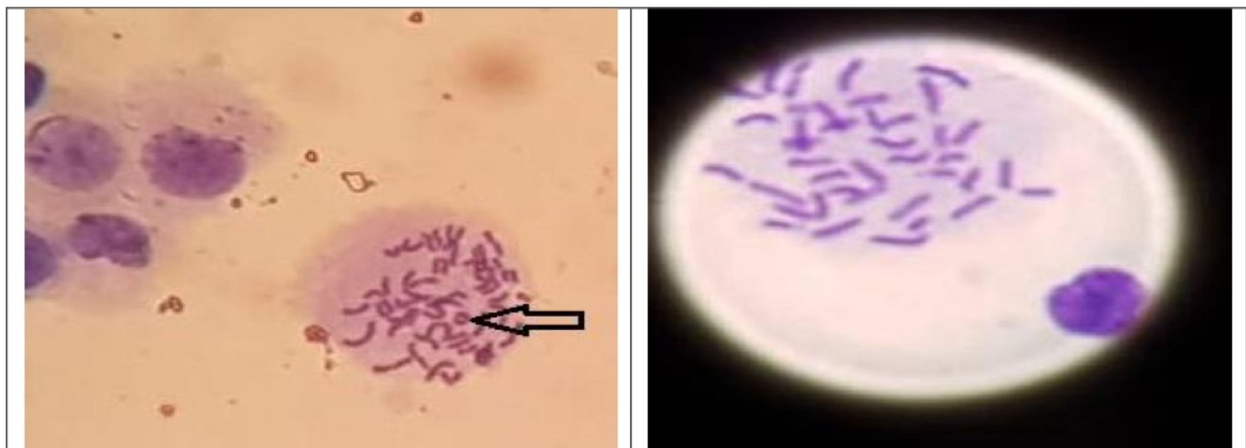

Fig. 2: Effect of heavy metal on mice blood lymphocytes exposed to cadmium and showing the damage of genetic material (ring chromosome).

In the image on the left, the mitotic index (MI) and blast index (BI) are observed in mice exposed to cadmium, along with detectable alterations in genetic material, including the formation of ring chromosomes. In contrast, the image on the right shows no observable genetic damage in the control group. The simplest approach for collecting comet assay data is to determine the proportion of cells exhibiting DNA migration. However, this method is highly dependent on electrophoresis conditions, which may influence the extent of DNA damage detected. Table (1) indicates positive comet assay results in several blood samples, even at relatively low cadmium exposure levels, suggesting the presence of DNA strand breaks in exposed animals. Comet assay classification is useful for quantifying DNA damage based on parameters such as tail length and the percentage of DNA in the tail. By assigning cells to

different migration categories, the extent of DNA damage in cells from culture media or animal tissues can be quantitatively assessed.

The comet assay commonly uses the parameter of DNA migration length, measured in micrometers. This parameter is believed to have a direct relationship with the size of migrated DNA fragments and reflects the extent of single-strand breaks (SSBs) and alkali-labile sites, which are associated with DNA damage. To obtain measurements in micrometers, a microscope equipped with an ocular micrometer or a calibrated imaging system is used. This can be achieved either by placing a calibrated scale over images of positive and negative control cells or by using a digital camera connected to the microscope. During image analysis, several criteria are applied to define the limits of DNA migration. The term "tail length" is commonly used to describe the extent of migrated DNA in comet images, although some researchers may use alternative terminology. Measurements may also include ratios such as tail length to head size or DNA distribution parameters. Cells with no detectable DNA migration typically exhibit a ratio close to one. Reactive oxygen species (ROS) are known to induce damage to large biomolecules through oxidative stress. Such damage is associated with aging-related diseases, including cardiovascular diseases, Parkinson's disease, diabetes, and mitochondrial disorders [42]. The relationship between ROS production and cellular damage forms the basis of the free radical theory of aging. Elevated environmental concentrations of heavy metals, including zinc and cadmium, may contribute to cellular damage by promoting oxidative stress and free radical formation, leading to molecular and DNA damage. Cadmium (Cd) is a heavy metal with no known biological function and is widely distributed in the environment due to both natural processes and anthropogenic activities [43]. It has been classified as a human and animal carcinogen; however, its exact mechanisms of toxicity are still not fully understood [44].

Cadmium chloride ($CdCl_2$) has been reported to induce DNA single-strand breaks, DNA–protein cross-links, and chromosomal aberrations, particularly in V79 Chinese hamster cells [45]. As one of the most toxic and widespread environmental contaminants, cadmium is recognized as a carcinogenic metal in mammals [46].

The mechanisms underlying cadmium-induced teratogenicity and genotoxicity are complex and may involve multiple molecular targets. Cadmium can interfere with DNA repair processes through zinc ion substitution in DNA repair enzymes [47], directly interact with DNA bases such as adenine and guanine leading to DNA adduct formation [48], and alter the expression of transcription factors involved in apoptotic pathways [48]. In addition, cadmium can bind to antioxidant molecules such as ascorbate and glutathione, reducing their scavenging capacity and increasing reactive oxygen species (ROS) levels, which subsequently damage DNA [49].

Several studies support these findings. For example, occupational exposure assessments using the comet assay in battery workers in Poland demonstrated that chronic cadmium exposure results in DNA damage, including single-strand breaks (SSBs) and alkali-labile sites (ALS) [50]. Similarly, cadmium exposure has been associated with various genotoxic effects, including DNA damage and chromosomal aberrations [51].

Although the precise mechanism of cadmium toxicity is not yet fully clarified, experimental studies have shown that cadmium chloride is highly cytotoxic to human lens epithelial cells. In vitro studies further indicate that cadmium induces free radical–mediated DNA damage in bacteria, leading to single-strand breaks and disruption of nucleic acid and protein synthesis [52].

The results of the present study demonstrated the induction of micronuclei in mouse lymphocytes following exposure to different concentrations of cadmium. In agreement with our

findings, several previous studies have reported positive results in micronucleus tests conducted on animal lymphocytes and other tissue cells after cadmium administration.

Cadmium pollution is recognized as a major environmental problem that can disrupt a wide range of physiological and biochemical processes [53]. The formation of micronuclei in blood lymphocytes following exposure to cadmium, as well as other heavy metals such as copper and chromium, supports earlier findings indicating their cytotoxic and genotoxic effects in mammals [54].

Micronuclei (MN) are widely accepted biomarkers of genotoxic damage and chromosomal instability. DNA damage events can be simultaneously evaluated using the cytokinesis-block micronucleus cytome (CBMNcyt) assay. This method is considered one of the most reliable and widely used tools for assessing environmental mutagenicity due to its simplicity, efficiency, and rapidity [55]. The results of the present study indicate that cadmium exhibits clear mutagenic activity. However, the exact mechanisms underlying micronucleus formation are not yet fully understood. Fenech [56] proposed that micronuclei may arise from the misrepair of DNA double-strand breaks, leading to chromosomal fragments or whole chromosomes that fail to be incorporated into daughter nuclei during telophase. This may result from defects in spindle attachment or errors during chromosome segregation in anaphase.

Inhibition of DNA repair is considered an important mechanism of cadmium (Cd)-induced genotoxicity. In the present study, this effect was demonstrated through changes in micronucleus (MN) frequency and mitotic index (MI). It has been proposed that cadmium exerts its genotoxic effects by competing with zinc for common binding sites on enzymes involved in DNA synthesis and repair [57].

A variety of genotoxicity assays have shown that Cd induces DNA damage in a concentration-dependent manner [58]. In the current investigation, exposure of mice to cadmium also resulted in increased frequencies of nuclear abnormalities, including ring chromosomes, as illustrated in Figure (1). Similar findings have been reported by several authors, who observed that chemical and heavy metal exposure induces nuclear abnormalities in different mouse tissues [59].

The present study further revealed that the highest frequencies of micronuclei (MN) and nuclear abnormalities were recorded after three months of exposure to cadmium. MN frequencies in erythrocytes increased in both a dose- and time-dependent manner during the exposure period [60]. Likewise, previous studies have reported similar dose- and time-dependent increases in micronucleus induction in peripheral blood cells. Genotoxic studies on organisms exposed to heavy metal–contaminated environments have consistently demonstrated DNA strand breaks, supporting the use of animals as sensitive indicators of environmental genotoxicity and mutagenicity [61].

Cadmium is recognized as a significant environmental toxicant among heavy metals due to its ability to accumulate in living organisms, posing serious risks to both environmental and public health.

Additionally, the present study indicated that mitotic index (MI), blast index (BI), and micronucleus (MN) frequencies reached their highest levels after three months of exposure to cadmium (and zinc, if included in the experimental design). These parameters showed a gradual increase starting from the second month, with both dose- and time-dependent trends observed throughout the exposure period.

Conclusion

The results of the present study demonstrated the induction of genotoxic damage, as evidenced by changes in the mitotic index (MI), blast index (BI), and micronucleus (Mn)

frequency in mice exposed to cadmium and zinc. The use of mice as a model for studying the effects of pollution is of increasing importance, as it allows for the early detection of environmental contamination. These organisms respond to toxic agents in a manner similar to that of higher vertebrates, which enables the assessment of substances that may pose potential risks to human health.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

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تقييم التأثيرات السُميّة الخلوية للمعادن الثقيلة في الحيوانات المختبرية

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الخلاصة:

تشير الأدلة العلمية إلى أن التعرض للمعادن الثقيلة، التي تنتشر في المنتجات الاستهلاكية والمياه والهواء، قد يؤدي إلى تأثيرات سمّية خلوية وجينية خطيرة. هدفت هذه الدراسة إلى تقييم التأثيرات المشتركة للتعرض لكلٍّ من الزنك (Zn) والكادميوم (Cd)على التركيب الوراثي لفئران المختبر البيضاء .(C57BL) وقد ثبت أن المعادن الثقيلة، ولا سيما الكادميوم، تُحدث أضرارًا في الحمض النووي لدى الإنسان والحيوان.

تم تقسيم 150 فأرًا إلى ثلاث مجموعات متساوية، حيث عُرضت المجموعة الأولى إلى أملاح الكادميوم بتركيز 0.685 ملغم/لتر في مياه الشرب، والمجموعة الثانية إلى أملاح الزنك بتركيز 0.567 ملغم/لتر، في حين استُخدمت المجموعة الثالثة كمجموعة سيطرة. استمرت المعاملة لمدة 90 يومًا. ولتقييم التأثيرات السمية الخلوية والجينية، استُخدمت اختبارات المذنب (Comet assay)، ومؤشر الانقسام الخيطي(MI) ، ومؤشر الأرومات (BI) ، واختبار النوى الدقيقة (MN) في الخلايا اللمفاوية.

أظهرت النتائج أن التعرض المزمن للكادميوم أدى إلى زيادة معنوية في مؤشر الانقسام الخيطي (37.67 ± 4.95) وتكرار النوى الدقيقة (1.42 ± 0.26) عند مستوى دلالة($P < 0.01$) ، مع تسجيل أعلى القيم في الشهر الثالث من التجربة. في المقابل، لم تُظهر مجموعة الزنك فروقًا معنوية مقارنة بمجموعة السيطرة. وتؤكد هذه النتائج السمية الجينية للكادميوم من خلال إحداثه تغيرات خلويه ملحوظة. وعليه، تبرز أهمية الرصد المستمر للتعرض البيئي للمعادن الثقيلة لتقييم المخاطر المحتملة على الصحة والسلامة الوراثية للكائنات الحية.

الكلمات المفتاحية: الانوية الدقيقة ، معامل الانقسام الخلوي، معامل الأرومات اللمفاوية ، الكادميوم ، الخلايا اللمفاوية.