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Application of the *Allium* Test in Toxicity Studies of Lead and Copper: A Cytological Perspective

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Abstract: The *Allium* test is a cytological method used to monitor the impact of heavy metals. It can be used to evaluate meristematic tissues and highlight abnormalities occurring during mitotic division, with the advantage of being both rapid and economical. Copper and lead are among the most widespread metals in everyday life, mainly due to the worldwide expansion of industrialization, and are present in soil, water, and air. Using *Allium sativum* as a bioindicator for this study, statistical analysis confirmed significant differences in genotoxicity between the two metals, reflected by the inhibition of mitotic activity (MI) or increased indices of cellular abnormalities (AI). Toxicity was dose- and time-dependent for both metals, with copper exhibiting greater genotoxic effects than lead. Copper caused a significant reduction in MI, even at relatively low concentrations, with the IC_{50} observed at 0.50 mM after 72 h of exposure. In contrast, for lead, the IC_{50} was recorded from 0.75 mM after 72 h exposure. The advantages of the *Allium* test were demonstrated by its simplicity and high sensitivity in detecting abnormalities. In our experiment, chromosome abnormalities such as chromosome bridges, as well as isolated, delayed, or sticky chromosomes, were observed. In addition, at a concentration of 0.25 mM for copper (72 h exposure) and 0.50 mM for lead (72 h exposure), cellular abnormalities, including giant cells and binucleated cells, were identified.

Keywords: mitotic index (MI); abnormalities index (AI); IC_{50}; heavy metals; genotoxicity

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1. Introduction

Heavy metal pollution contributes to the deterioration of human health by increasing mortality and reducing life expectancy [1,2]. Non-biodegradability represents a biological threat due to its adverse effects on cellular integrity and metabolic processes. An estimated 47,000 people die every year from heavy metal pollution in Poland, the European county with the third highest levels of air pollution, after Bulgaria and Cyprus [3]. In the agricultural ecosystem, excess heavy metals in the soil severely affect the health of crop plants, compromising their yield and quality [4–7]. Since even small concentrations of heavy metals can determine metabolic modifications, the maximum allowable limits are

of great importance to public health [8]. Heavy metals binding to functional groups of enzymatic proteins, such as sulfhydryl (-SH) groups [9], inhibit essential processes such as glycolysis and the Krebs cycle [10]. Also, the impact of heavy metals depends greatly on the genotypes, the stage of development, and age [11–13].

The primary pathway through which plants accumulate heavy metals is through root absorption from the soil [14–16]. Since heavy metals are non-biodegradable, they can accumulate in plants, which can subsequently be consumed, posing a significant risk to human health [17]. The effect in plants is reflected in physiological processes such as photosynthesis [18], nutrients absorption [19], and root system development by accumulation in the plant's tissues. Furthermore, cellular functions such as cell division are effected [20], leading to morphological deformation and a dramatic increase in the induction of programmed cell death (PCD) [21,22].

Cellular damage resulting from heavy metals is primarily caused by the generation of reactive oxygen species (ROS). In plants, ROS production has also been demonstrated under the impact of drought and salinity. ROS are molecules containing oxygen atoms with unpaired electrons, formed as byproducts of incomplete decomposition processes [23]. The effect on the mitotic index (MI) and the significant increase in the abnormalities index (AI) correlate with ROS production, as both have been demonstrated in several studies involving heavy metals [23].

The presence of heavy metals in soil not only influences or reduces crop production but also affects water quality, exacerbates the effects of climate change, and poses significant threats to human health [24,25]. According to reports on soil contamination, approximately 70% of heavy metal pollution is attributed to industrial and mining activities [26]. The most common heavy metals are represented by copper (Cu^{+2}) and lead (Pb^{+2}). Cu^{+2} and Pb^{+2} rank among the most significant and frequently associated soil contamints, posing a serious concern for the health of the plants and the ecosystems [27,28].

Lead (Pb^{+2}), a heavy metal non-essential to the organism, continues to be widely used in numerous industrial processes and is found as a contaminant in all environmental compartments [29]. Pb^{+2} absorbed by roots becomes bound to the carboxyl groups of uronic acid mucilage or directly to the polysaccharides on the surface of rhizodermal cells [30]. Once adsorbed on the rhizodermal surface of roots, lead can passively enter the roots through water transport pathways [31]. One study found that the highest concentrations are found in the root tips, where the cells are young and have thin cell walls [32]. According to studies, Pb^{+2} is typically found in soil at normal concentrations ranging from 10–40 mg/kg (≡0.048–0.193 mM). The legislative limits for lead (Pb^{+2}) across individual EU countries vary widely, ranging from 45 to 1200 mg/kg DM (≡0.217–5.792 mM) [33–35].

Copper (Cu^{+2}), a metal essential for the body, is required for the organism's metabolic processes in the range of 1.4 to 2 mg per kilogram of body weight [36]. Cu^{+2} is not only essential for the organism but is also widely used in agriculture; e.g., copper sulfate has proven to be an excellent fungicide against mildew [37] and other plants diseases [38]. The widespread use of copper in agriculture as a fungicide or fertilizer, as well as in the electrical industry and for water conduits, has highlighted the need for timely prevention of its toxic concentrations [39]. Although an essential metal, a Cu^{+2} intake of 10–20 mg/day can cause symptoms of toxicity, while very high doses exceeding 60 mg/day can lead to liver failure and in some cases, even death [39]. The legislative limits for copper across individual EU countries range from 75 to 1750 mg/kg DM (≡1.180 mM–27.539 mM) [35]. Taking into consideration the wide variability in limits across different countries regarding heavy metals, there is a need for an early warning system [34,40–43]. Utilizing bioindicators, such as the *Allium* test, can serve as an effective tool for preventing soil contamination [44]. This approach enables the detection of concentrations of heavy metals and other chemicals that

may not yet visibly affect plants but which could compromise their physiological processes, such as nutrient absorption and root system development [45].

The *Allium* test was used as a bioindicator to monitor the genotoxicity of several heavy metals [46–48]. The advantages of using the *Allium* test for cytological analyses are significant and make it appropriate as a bioindicator for heavy metals toxicology [49–51]. The *Allium* test is a short-term test that offers the ability to monitor cellular processes such as mitotic activity and chromosomal aberrations, thereby evaluating DNA integrity under specific conditions or treatments [52]. It is widely used due to its cost-effective advantages for assessing potential risks of heavy metals in species like *Allium cepa* [53] but also in *Allium sativum* [54]. Another considerable advantage of the *Allium* test is its high sensitivity, demonstrated through changes in the mitotic index rate or the occurrence of chromosomal aberrations [34,55–57]. Furthermore, due to the large size of the cells and the easily observable chromosomal structures under a microscope, the test provides clear evidence of genotoxicity at specific concentrations of heavy metals [34,55,56]. Studies demonstrate that applying the *Allium* test reveals modifications in mitotic division even at low concentrations, enabling the monitoring of various cellular abnormalities. These abnormalities include chromosomal bridges, lagging chromosomes, stickiness, binucleated cells, nuclear lesions, giant cells and c-mitosis [52].

In this study, we aimed to (i) investigate the cytological impact of lead (Pb^{+2}) and copper (Cu^{+2}) on the mitotic divisions of *Allium sativum* L., focusing on parameters such as the mitotic index (MI) and the abnormalities index (AI), (ii) determine the concentration of lead and copper that inhibits the mitotic process by 50% (IC_{50}), (iii) compare the different genotoxic effects of lead and copper, and to (iv) analyze the correlations between multiple cellular indices and parameters under exposure to heavy metals.

2. Materials and Methods

2.1. Chemicals

Copper sulfate ($CuSO_4$) was purchased from SC GXG Chemicals SRL (Râmnicu Vâlcea, Romania), and lead acetate $Pb(C_2H_3O_2)_2$ was obtained from LiMac Science (Kekava, Latvia), both with the highest available purity.

2.2. Plant Material Used

For the experiment, the *Danubius* garlic variety, a Romanian-origin variety certified by the Gene Bank of Buzău, was used [34,58]. The evaluation of the effects of the heavy metals Cu^{2+} and Pb^{2+} was conducted using equal-sized bulbs of *Allium sativum* L. (2n = 16).

Prior to the experiment, the bulbs of *Allium sativum* L. were kept in a cool room (4 °C) with light for approximately two weeks to synchronize their biological functions [59].

2.3. Experimental Design

For the study, five repetitions of each treatment of heavy metals solutions were used.

In the initial phase of the experiment, the garlic bulbs were germinated for 72 h at a temperature of 18–22 °C in distilled water.

After germination, the germinated bulbs with roots of similar length were transferred to the corresponding experimental variants.

Prior to treatment, the germinated bulb root tips were removed to obtain a complete image of the mitotic activity after the treatments.

The experimental treatments consisted of distilled water (control) and solutions of $CuSO_4$ and $Pb(C_2H_3O_2)_2$ at concentrations ranging from 0.25 mM to 1.00 mM.

During the treatment, secondary roots were collected in the morning, at 11:00 AM and 12:00 AM, after 24, 48, and 72 h [60] and placed in Eppendorf tubes in the fixative solution until the start of the cytological analyses.

The collected roots were subjected to the fixation, hydrolysis, and staining of biological materials required for microscopic analysis [61,62].

2.4. Treatments Used and Preparations

For the study, four concentrations of Cu^{2+} and Pb^{2+} were used to determine the cytological effects of the heavy metals. Stock solutions of 100 mM concentration of $CuSO_4$ and $Pb(C_2H_3O_2)_2$ were prepared and subsequently diluted with distilled water (dH_2O) to obtain treatment solutions of 0.25 mM, 0.50 mM, 0.75 mM, and 1 mM. These four treatments were applied to the germinated garlic bulbs in distilled water to evaluate the cytological impact of each metal.

2.5. Fixation, Hydrolysis, and Staining Procedures

Secondary roots collected after 24, 48, and 72 h of each treatment were examined under the microscope for mitotic division assessment.

Fixation was conducted using Carnoy's reagent, obtained by preparing the solution in a 3:1 ratio of ethyl alcohol (96%) and glacial acetic acid. The fixation was performed in an alcohol–acetic acid mixture for 1 h at a low temperature of 2–4 °C [61,62]. Preservation was employed until complete cytological analysis. To avoid over-fixation, the roots were stored in a solution of 70% ethyl alcohol at a low temperature of 2–4 °C. To remove the fixation solution, 1N HCl was used at room temperature for 5 min.

To facilitate the effective staining of the cellular structures, the collected roots were subjected to hydrolysis using 2–3 mL of 1N HCl for 7–8 min in a water bath at 60 °C. After hydrolysis, the solution was removed using filter paper.

For staining the collected biological material, the roots were kept in Carnoy's fixative for 24 h using the Feulgen method [63,64].

2.6. Microscope Slide Preparation and Microscope Used

For the preparation of the microscope slides, the biological material, after undergoing fixation, hydrolysis, and staining, was spread on a microscope slide to enable the observation and monitoring of mitotic activity and the impact of the treatments used. Using the squash method, the following steps were performed [65]:

(a) A drop of Carnoy's reagent was applied to the microscope slide.
(b) The root tip (1–2 mm) was placed into the drop.
(c) The slide was gently heated over the flame of a spirit lamp.
(d) A cover slip was placed over the specimen.
(e) The cover slip was pressed to uniformly spread the material across the slide.
(f) Excess stains were removed using filter paper by gently pressing.

The microscope slides were analyzed to determine the mitotic index (MI) and abnormalities index (AI) of the experimental samples. The analysis was conducted using an Optika microscope at 400× magnification under normal light. Microscopic photographs were captured using the digital camera attached to the microscope.

2.7. Cytological Assessment

For cytological analyses, the mitotic rate for each root was calculated after examining ten microscopic fields. The cytological analyses involved evaluating the mitotic index of

the meristematic tissues at the root tip of each experimental sample. The mitotic index (MI) was expressed as the ratio of dividing cells to the total number of cells.

$$\text{Mitotic index (MI)} = \frac{Number\ of\ cells\ in\ division}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (1)$$

During our study, the number of cells in interphase (Figure 1A) was also monitored. Additional analyses involved monitoring the percentage of cells in prophase (Figure 1B), metaphase (Figure 1C), anaphase (Figure 1D), and telophase (Figure 1E).

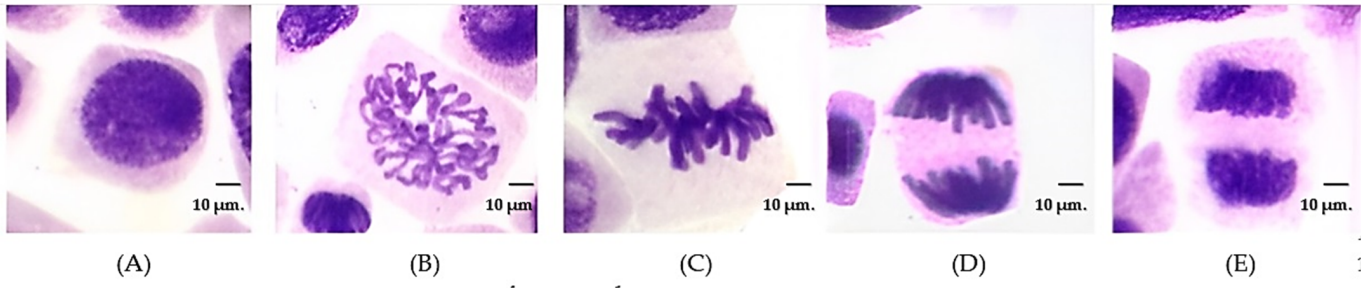

Figure 1. *Allium sativum* cells at different stages: interphase (**A**), prophase (**B**), metaphase (**C**), anaphase (**D**), and telophase (**E**) (Optika microscope, magnification: 400×, original images from the Genetics Department).

For calculating the interphase index (Ii), prophase index (Ip), metaphase index (Im), anaphase index (Ia), and telophase index (It), the following calculation formulas were used:

$$\text{Interphase index (Ii)} = \frac{Number\ of\ interphase\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (2)$$

$$\text{Prophase index (Ip)} = \frac{Number\ of\ prophase\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (3)$$

$$\text{Metaphase index (Im)} = \frac{Number\ of\ metaphase\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (4)$$

$$\text{Anaphase index (Ia)} = \frac{Number\ of\ anaphase\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (5)$$

$$\text{Telophase index (It)} = \frac{Number\ of\ telophase\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (6)$$

Cytological evaluation of the heavy metals was also conducted, during which cell abnormalities and cells deviating from normal characteristics were monitored (Figure 2).

In the study, cellular abnormalities were observed at all stages of cell division, including aberrations such as chromosomal bridges (Figure 2a), unevenly distributed metaphases (Figure 2b), isolated chromosomes in anaphase (Figure 2c), isolated chromosomes in metaphase (Figure 2d), sticky chromosomes (Figure 2e), lagging chromosomes (Figure 2f,g), and additional instances of lagging chromosomes (Figure 2k) [57].

Furthermore, aside from the anomalies during the mitotic division process, cellular abnormalities were also observed during the interphase, when the cell was not undergoing mitotic division, such as binucleated cells (Figure 2i) and giant cells (Figure 2j).

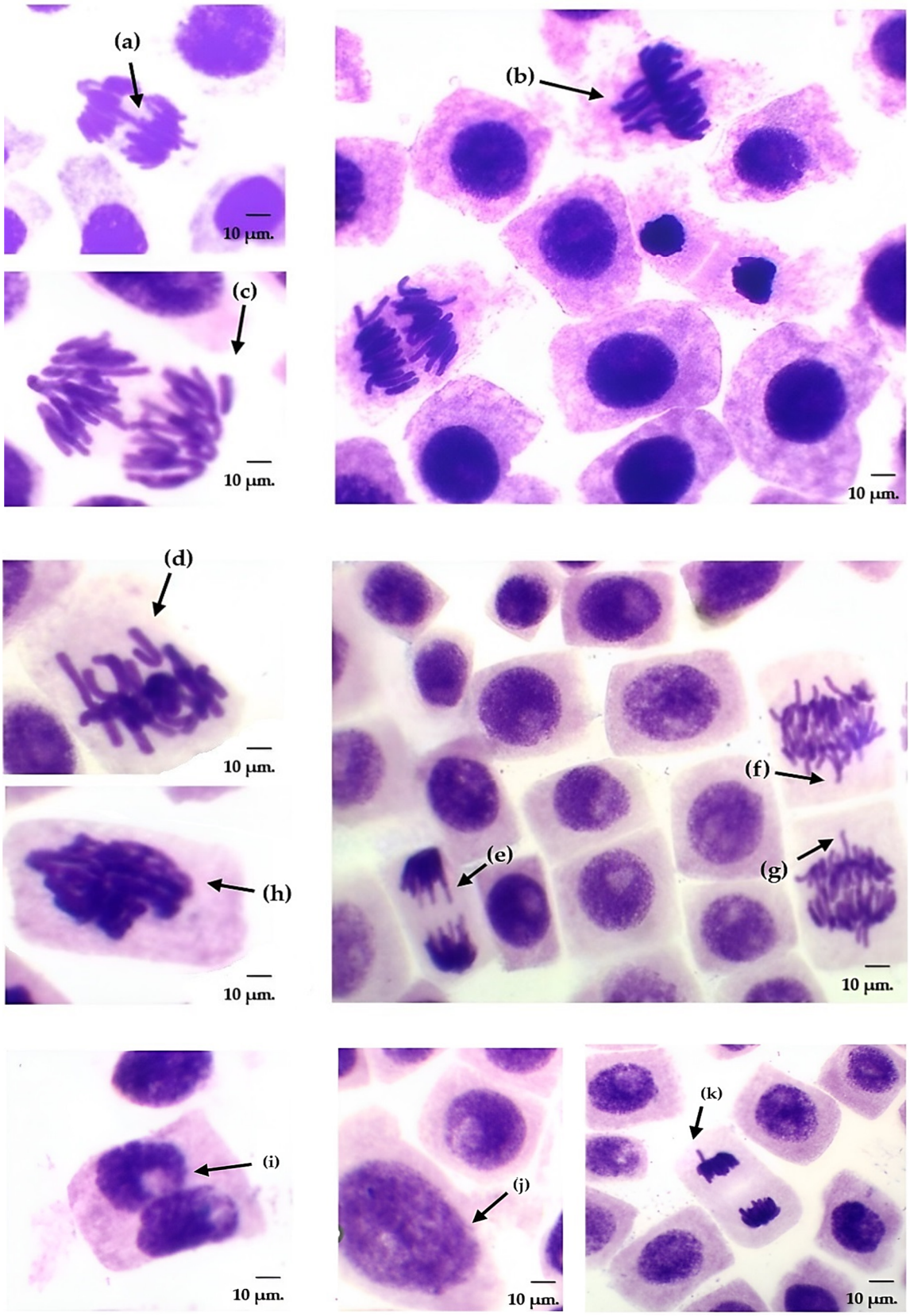

Figure 2. *Allium sativum* cells that exhibit abnormalities and deviate from normal characteristics: (**a**) chromosomal bridges, (**b**) unevenly distributed metaphases, (**c**) isolated chromosomes in anaphase, (**d**) isolated chromosomes in metaphase, (**e**) sticky chromosomes, (**f**,**g**) lagging chromosomes, (**h**) sticky metaphase, (**i**) binucleated cells, (**j**) giant cells, and (**k**) lagging chromosomes (Optika microscope, magnification: 400×, original images from the Genetics Department).

To calculate the abnormalities index (%), the number of abnormal cells was quantified relative to the total number of cells evaluated in the microscopic field (1).

$$\text{Abnormalities index (AI)} = \frac{Number\ of\ abnormal\ cells}{Total\ number\ of\ cells\ anlyzed} \times 100 \quad (7)$$

After determining the mitotic indices and the abnormalities index for all experimental variants, the data were consolidated based on dose and time to evaluate how these indices were influenced. The data were recorded and subjected to statistical analysis to determine the differences in genotoxicity between Cu^{+2} and Pb^{+2}.

2.8. Determination of the Inhibition Index (IC_{50})

The determination of the 50% inhibitory concentration (IC_{50}) for each experiment sample was calculated based on the mitotic index of the control sample (dH_2O) at 24 h.

The mitotic index value of the control sample at 24 h served as a reference, enabling the determination of IC_{50} values for each experimental treatment and time of exposure. This approach allowed the inhibitory effects on cell division to be highlighted, providing the possibility of comparisons between treatments and the expression of IC_{50} concentrations for Cu^{+2} and Pb^{+2} [66,67].

2.9. Statistical Analysis

Statistical analyses were performed using RStudio software v4.4.2, which was used to conduct ANOVA tests to determine the differences and influence of the metal type and treatment period on the mitotic index and abnormalities index, at a significance level of $p < 0.05$. The Tukey test was performed to determine the significant differences between Cu^{+2} and Pb^{+2} effects. To evaluate the impact of heavy metal treatments on the mitotic index, a polynomial regression analysis was applied. A heatmap was generated based on Pearson correlation coefficients derived from the cytological idicator results of the *Allium* test on each tratment. The measured variables were standardized using the Z-score standardization method, implemented in RStudio software. Two datasets were analyzed—one for copper (Cu) treatments and the other for lead (Pb) treatments.

3. Results

3.1. Analysis of the Effect of Lead on the Mitotic Index

The ANOVA test results presented in Table 1 highlight the significant influence of dose, time, and their interaction on the mitotic index under lead exposure conditions.

Table 1. ANOVA results showing the effects of dose, time, and their interaction on the mitotic index under lead exposure.

Source	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Dose	4	344.3	86.09	86.41	$<2 \times 10^{-16}$ ***
Time	2	48.4	24.22	24.31	1.85×10^{-8} ***
Dose–Time	8	5.70	0.71	0.71	0.68
Residuals	60	59.8	1.00		

Significance level: *** $p < 0.001$.

The effect of lead on the mitotic index shows a highly significant statistical relevance ($p < 0.001$), indicating that increasing lead dose results in significant alterations in mitotic activity.

Another critical factor influencing the reduction in the mitotic index is time, with an equally significant statistical impact ($p < 0.001$). This underscores that the duration of exposure significantly affects the mitotic index.

The interaction between dose and time is not statistically significant ($p > 0.05$) (Table 1). This indicates that the genotoxic effect of lead at the analyzed doses on the mitotic index does not show an increasing severity based on the duration of exposure. The absence of an interaction between the two factors (dose and exposure time) suggests that this does not occur. This study may reflect the consequence that lead has a cumulative rather than a chronic effect on the cell, with its effects being significant over time.

The results of the cytological analyses presented in Table 2 show an inhibitory effect of concentration for different lead concentrations and exposure times, based on the mitotic index and the abnormalities index.

The highest mitotic index (MI) values were observed in the control samples (dH_2O), reaching 11.82% at 24 h, indicating normal mitotic activity. On the other hand, as lead concentration and exposure time increased, varying levels of mitotic index inhibition were observed. At higher lead concentrations, the mitotic index decreased significantly, particularly at 1 mM, for which it reached its lowest level at 72 h (5.06%). This result suggests a progressive inhibitory effect of lead on cell division as concentration or exposure time increase.

With regards to the 50% inhibitory concentration relative to the control (IC_{50}), the results indicate that this level was reached at concentrations of 0.75 mM and 1 mM after 72 h of exposure. With regards to the abnormal index (AI), no abnormal cells were detected in the control samples. The same result applied to samples treated with 0.25 mM lead. However, the first abnormalities were observed at a concentration of 0.50 mM after 72 h of exposure.

This underlines the importance of exposure time in the development of chromosomal abnormalities. Furthermore, the genotoxic effect of lead became more pronounced at higher doses and longer exposure times. As lead concentration and exposure time increased, the abnormality index (AI) rose significantly, reaching 1.81% at 1 mM after 72 h (Table 2).

The effect of long-term lead exposure is highlighted by polynomial analyses. The mitotic index (MI) for the control sample remains relatively constant over time, reflecting normal cell division in the absence of lead. Similarly, at a low lead concentration of 0.25 mM, no significant changes were observed, demonstrating that at this concentration, lead does not significantly inhibit cell division during the studied exposure period (Table 2 and Figure 3).

On the other hand, at a concentration of 0.50 mM, a reduction in mitotic division capacity becomes evident, indicating the onset of inhibition and a decrease in the mitotic index.

The inhibitory effect is most pronounced at higher concentrations of 0.75 mM and 1.00 mM, where a strong reduction in the mitotic index is observed, closely related to the lead concentration and exposure time ($R^2 = 0.78$).

This result reflects the genotoxic effect of lead at doses of 0.75 mM and 1 mM during prolonged exposure periods, leading to a significant decrease in mitotic activity.

Table 2. The results of cytological analyses and the calculation of the inhibitory concentration for various lead concentrations.

Dose	Time	Mitotic_Index (%)	IC *	Abnormalities_Index (%)	Interphase (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)
Control	24	11.82 ± 0.42 a	-	0 ± 0 b	88.18 ± 0.42	5.62 ± 0.71	2.28 ± 1.04	1.31 ± 0.53	1.51 ± 0.34
Control	48	11.35 ± 0.59 a	-	0 ± 0 b	88.65 ± 0.59	6.24 ± 0.8	1.7 ± 0.41	1.58 ± 0.48	1.83 ± 0.7
Control	72	10.99 ± 0.49 ab	-	0 ± 0 b	89.01 ± 0.49	6.02 ± 0.82	2.25 ± 0.7	1.12 ± 0.42	1.6 ± 0.49
0.25 Mm	24	11.52 ± 1.05 a	2.54	0 ± 0 b	88.48 ± 1.05	6.1 ± 0.81	1.96 ± 0.72	1.51 ± 0.68	1.96 ± 0.46
0.25 Mm	48	10.97 ± 1.45 ab	7.19	0 ± 0 b	89.03 ± 1.45	6.17 ± 1.03	1.85 ± 0.74	1.5 ± 0.63	1.44 ± 0.48
0.25 Mm	72	9.59 ± 1.2 abc	18.87	0 ± 0 b	90.41 ± 1.2	5.66 ± 1.18	0.99 ± 0.33	1.46 ± 0.3	1.48 ± 0.35
0.5 mM	24	10.32 ± 1.34 ab	12.69	0 ± 0 b	89.68 ± 1.34	7.31 ± 1.16	0.72 ± 0.1	0.72 ± 0.45	1.56 ± 0.46
0.5 mM	48	8.83 ± 1.47 bcd	25.3	0 ± 0 b	91.17 ± 1.47	6.23 ± 1.74	0.8 ± 0.51	0.51 ± 0.42	1.28 ± 0.74
0.5 mM	72	7.64 ± 1.04 cde	35.36	0.69 ± 0.85 ab	92.36 ± 1.04	4.42 ± 0.7	1.01 ± 0.45	0.95 ± 0.53	1.26 ± 0.28
0.75 mM	24	7.60 ± 0.4 de	35.70	0 ± 0 b	92.40 ± 0.4	5.13 ± 1.11	1.08 ± 0.7	0.88 ± 0.53	0.51 ± 0.42
0.75 mM	48	6.81 ± 0.62 def	42.39	0.62 ± 0.94 ab	93.19 ± 0.62	3.32 ± 0.54	1.22 ± 0.57	0.97 ± 0.54	1.29 ± 0.29
0.75 mM	72	5.45 ± 0.51 ef	53.89	0.97 ± 0.67 ab	94.55 ± 0.51	3.19 ± 0.52	0.46 ± 0.39	0.86 ± 0.53	0.94 ± 0.51
1 mM	24	6.94 ± 0.55 def	41.29	0.67 ± 1.35 ab	93.06 ± 0.55	4.11 ± 0.56	1.04 ± 0.77	0.28 ± 0.35	1.51 ± 0.54
1 mM	48	6.26 ± 0.55 ef	47.04	1.37 ± 1.31 ab	93.74 ± 0.55	3.18 ± 0.81	1.42 ± 0.8	0.43 ± 0.54	1.23 ± 0.73
1 mM	72	4.74 ± 0.33 f	59.89	1.81 ± 1.06 a	95.26 ± 0.33	3.47 ± 0.49	0.3 ± 0.37	0.4 ± 0.52	0.57 ± 0.47

* Inhibitory concentration of the mitotic index: The reference value used was the mitotic index of 11.82 from the control sample at 24 h. Notes: The results for each column represent the means of five replicates, along with the standard deviations (±) for each index. Letters are used, according to the results of the Tukey pairwise comparison test, across columns to determine statistical significance. The highest values are denoted starting with the first letter, "a". Similarity between letters indicates non-significant differences between the values obtained ($p > 0.05$).

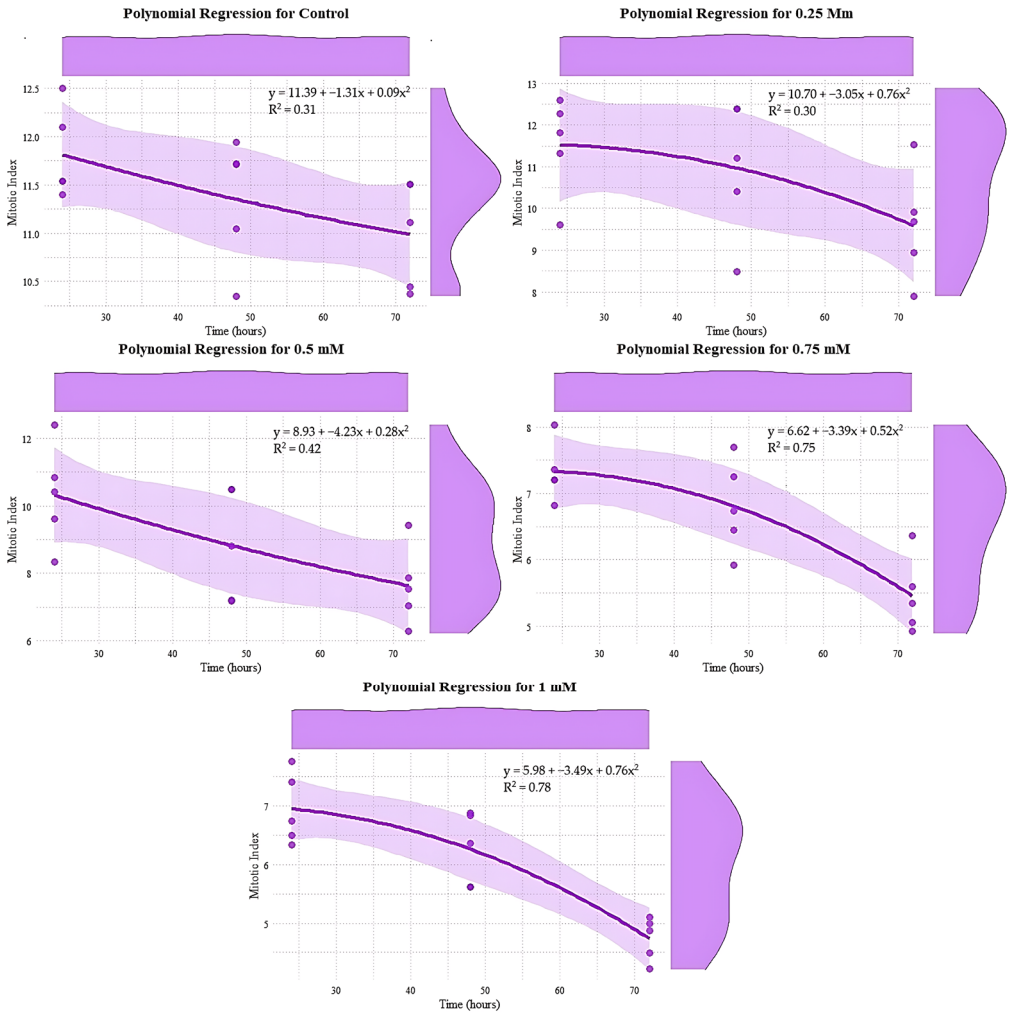

Figure 3. Polynomial regression analyzing the influence of time on the mitotic index under lead exposure.

3.2. Analysis of the Effect of Cu^{+2} on the Mitotic Index

The effect of copper observed in the mitotic index is substantially different, as it is significantly influenced by the dose and exposure time ($p < 0.001$). Furthermore, the interaction between dose and time is also significant ($p < 0.001$). Therefore, cellular division is inhibited and is dependent on both the dose used and the duration of exposure. Thus, the acute effect of copper is reflected, as increasing the dose leads to a more pronounced inhibitory effect on mitotic division over the exposure time (Table 3).

Table 3. ANOVA test for determining the factorial implications in the reduction of the mitotic index.

Source	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Dose	4	511.50	127.88	164.01	$<2 \times 10^{-16}$ ***
Time	2	106.10	53.04	68.02	3.75×10^{-16} ***
Dose–Time	8	25.2	3.15	4.05	6.78×10^{-4} ***
Residuals	60	46.80	0.78		

Significance level: *** $p < 0.001$.

According to the results regarding the effects of copper on the mitotic index, compared to the control the mitotic index (MI) decreases significantly with increasing copper concentration and exposure time (Figure 4).

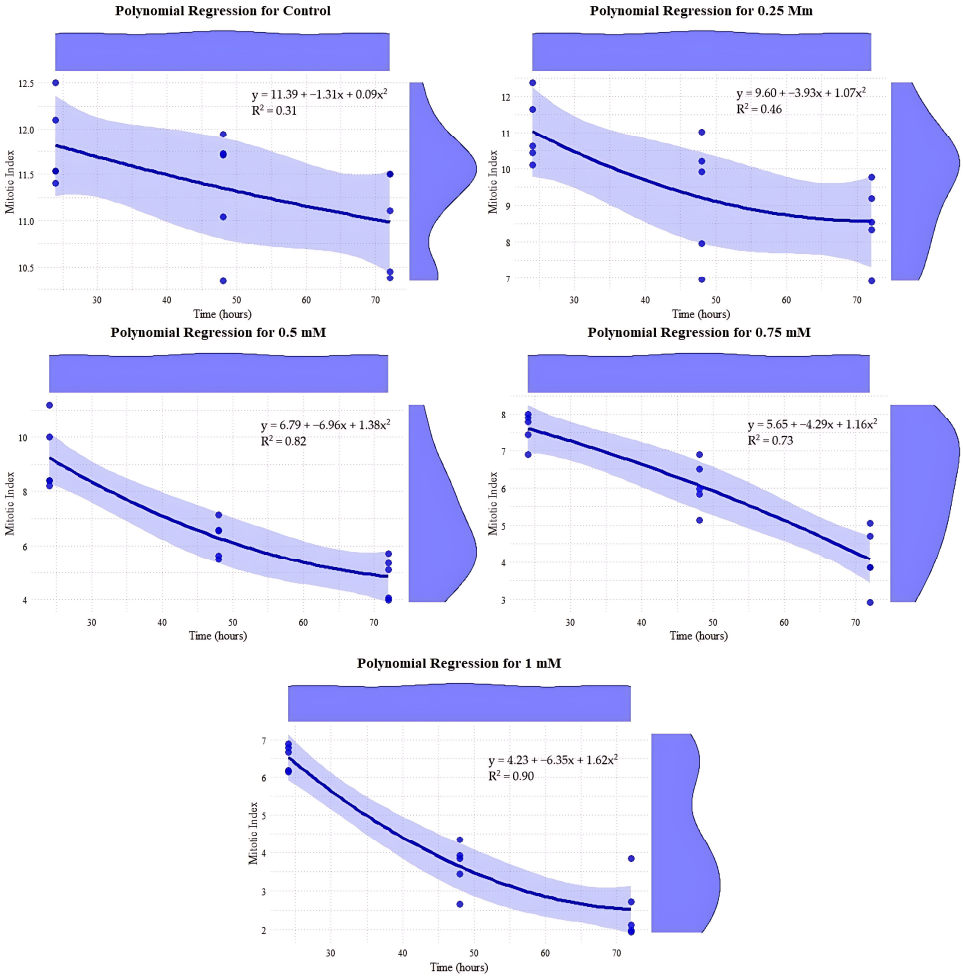

Figure 4. Polynomial regression showing the influence of time on the mitotic index under copper treatment.

For the control, the MI remains above 10%, regardless of the exposure duration. However, the MI decreases significantly at the first dose of 0.25 mM at a 48–72 h exposure time. Regarding the mitotic index, under copper treatment, the index was inhibited along with the concentration exposure.

The results indicate a significant inhibition of the mitotic index (MI) at the highest concentration (1 mM) and the longest exposure duration of 72 h. The reduction trend is significant, with only 2.52% of the cells in division (Table 4).

Regarding the 50% inhibitory concentration relative to the control (IC_{50}), the results indicate that this level was achieved at a concentration of 0.50 mM after 72 h of exposure, 0.75 mM after 72 h of exposure, and 1.00 mM after 48 h of exposure.

The observed inhibition in the MI highlights the genotoxic effects of copper on cellular division. Also, the decline in MI reflects the inhibition of cellular processes caused by the presence of numerous abnormal cells, which may disrupt chromosomal segregation during mitosis.

Abnormal cells were first detected at a concentration of 0.25 mM after 72 h of exposure. As concentration and exposure time increase, the abnormalities index (AI) increases significantly, revealing the genotoxic effect of copper. The highest AI was observed at the concentration of 1.00 after 72 h of exposure. This trend reveals copper's capacity to induce chromosomal abnormalities, highlighting its genotoxic effect, which could have long-term implications.

Table 4. The results of cytological analyses and the calculation of the inhibitory concentration for various copper concentrations.

Dose	Time	Mitotic_Index (%)	IC *	Abnormalities_Index (%)	Interphase (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)
Control	24	11.82 ± 0.42 a	-	0 ± 0 c	88.18 ± 0.42	5.62 ± 0.71	2.28 ± 1.04	1.31 ± 0.53	1.51 ± 0.34
Control	48	11.35 ± 0.59 a	-	0 ± 0 c	88.65 ± 0.59	6.24 ± 0.8	1.7 ± 0.41	1.58 ± 0.48	1.83 ± 0.7
Control	72	10.99 ± 0.49 ab	-	0 ± 0 c	89.01 ± 0.49	6.02 ± 0.82	2.25 ± 0.7	1.12 ± 0.42	1.6 ± 0.49
0.25 Mm	24	11.04 ± 0.84 ab	6.6	0 ± 0 c	88.96 ± 0.84	7.02 ± 0.14	1.33 ± 0.48	1.13 ± 0.32	1.55 ± 0.81
0.25 Mm	48	9.21 ± 1.51 bc	22.08	0 ± 0 c	90.79 ± 1.51	6.1 ± 1.06	1.51 ± 0.44	0.87 ± 0.78	0.73 ± 0.37
0.25 Mm	72	8.55 ± 0.96 c	27.66	0.49 ± 0.4 c	91.45 ± 0.96	5.35 ± 0.34	1.01 ± 0.59	1.4 ± 0.87	0.79 ± 0.48
0.5 mM	24	9.24 ± 1.18 bc	21.83	0.91 ± 1.02 bc	90.76 ± 1.18	6.45 ± 0.95	0.86 ± 0.52	1.22 ± 0.45	0.7 ± 0.35
0.5 mM	48	6.29 ± 0.62 de	46.79	1.74 ± 0.64 bc	93.71 ± 0.62	3.5 ± 1.07	1.23 ± 0.66	0.69 ± 0.35	0.87 ± 0.58
0.5 mM	72	4.84 ± 0.7 ef	59.05	2.07 ± 0.87 bc	95.16 ± 0.7	2.83 ± 0.43	0.95 ± 0.62	0.61 ± 0.31	0.45 ± 0.37
0.75 mM	24	6.79 ± 0.74 de	37.99	1.54 ± 1.39 bc	92.67 ± 0.4	4.67 ± 0.63	0.63 ± 0.33	0.76 ± 0.41	1.27 ± 0.35
0.75 mM	48	6.07 ± 0.6 de	48.65	1.88 ± 0.63 abc	83.78 ± 15.63	4.13 ± 0.9	0.63 ± 0.33	0.76 ± 0.41	1.27 ± 0.35
0.75 mM	72	4.07 ± 0.75 ef	65.57	2.45 ± 2.25 abc	95.93 ± 0.75	2.34 ± 0.94	0.19 ± 0.38	0.64 ± 0.54	0.9 ± 0.47
1 mM	24	6.54 ± 0.31 de	44.67	1.14 ± 0.96 abc	93.46 ± 0.31	3.53 ± 0.53	0.73 ± 0.7	1.13 ± 0.41	1.14 ± 0.46
1 mM	48	3.64 ± 0.57 fg	69.2	3.35 ± 1.31 ab	96.36 ± 0.57	2.55 ± 1.08	0.37 ± 0.45	0.37 ± 0.45	0.37 ± 0.45
1 mM	72	2.52 ± 0.72 g	78.68	4.23 ± 1.27 a	97.48 ± 0.72	1.3 ± 0.63	0.18 ± 0.36	0.3 ± 0.39	0.74 ± 0.44

* Inhibitory concentration of the mitotic index: The reference value used was the mitotic index of 11.82 from the control sample at 24 h. Notes: The results for each column represent the means of five replicates, along with the standard deviations (±) for each index. Letters are used, according to the results of the Tukey pairwise comparison test, across columns to determine statistical significance. The highest values are denoted starting with the first letter, "a". Similarity between letters indicates non-significant differences between the values obtained ($p > 0.05$).

Other mitotic indices, such as the prophase index, metaphase index, anaphase index, and telophase index, decrease with increasing concentration and prolonged exposure time (Table 4).

The results of the polynomial regression analysis highlight the trend of a decreasing mitotic index (MI) resulting from copper treatment. The MI is inhibited in a directly proportional manner, depending on the dose and exposure time, with a more pronounced effect observed at higher concentrations.

In comparison to control (dH_2O), where time has a minimal impact on the reduction in the MI, copper significantly inhibits cellular division, exhibiting a toxic effect. This inhibition becomes particularly evident starting at a concentration of 0.50 mM and intensifies up to 1.00 mM.

The increasing R^2 values with higher copper concentrations demonstrate a stronger predictability of the inhibitory effect as the dose increases. The MI trends toward zero, indicating a complete growth stop at higher concentrations (Figure 4).

3.3. Comparison Between Pb^{2+} and Cu^{2+} Regarding the Mitotic Index

According to the results, both metals reduce the capacity for mitotic division to varying degrees, highlighting the fact that certain metals, such as copper and lead in our case, have different mechanisms of action, inhibiting mitotic activity in distinct ways.

The results of the ANOVA test for the two heavy metals show a significant difference in their impact. This indicates that copper and lead influence mitotic activity differently, depending on the exposure time and the dose used (Table 5).

Table 5. ANOVA for the effects of type of metal treatment on the mitotic index.

Source	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Metal_Type	1	53.20	53.2	6.863	0.01 **
Residuals	148	1147.8	7.76		

Significance level: ** $p < 0.01$.

According to the results, the average mitotic index is lower for the copper treatments compared to lead treatments, indicating that copper has a stronger inhibitory effect on cellular division ($p < 0.05$) (Figure 5).

Figure 5. Boxplot showing the difference between the impact of Pb^{2+} and Cu^{2+} on the mitotic index.

3.4. Comparison of the Impact of Pb^{2+} and Cu^{2+} on the Abnormalities Index

The ANOVA test allowed us to compare the impact of different types of metals (Pb^{2+} vs. Cu^{2+}) on the abnormalities index (AI). The results obtained and presented in Table 6 highlight that there is a significant impact on and a notable difference between the results for the abnormalities index (AI), depending on the type of metal used ($p < 0.05$).

Table 6. ANOVA test for comparison of the impact of lead and copper on the abnormalities index.

Source	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Metal_Type	1	31.08	31.082	18.33	3.32×10^{-5} ***
Residuals	148	250.94	1.698		

Significance level: *** $p < 0.001$.

According to the results obtained, the number of cell abnormalities was higher under treatments based on copper compared to those based on lead ($p < 0.05$) (Figure 6).

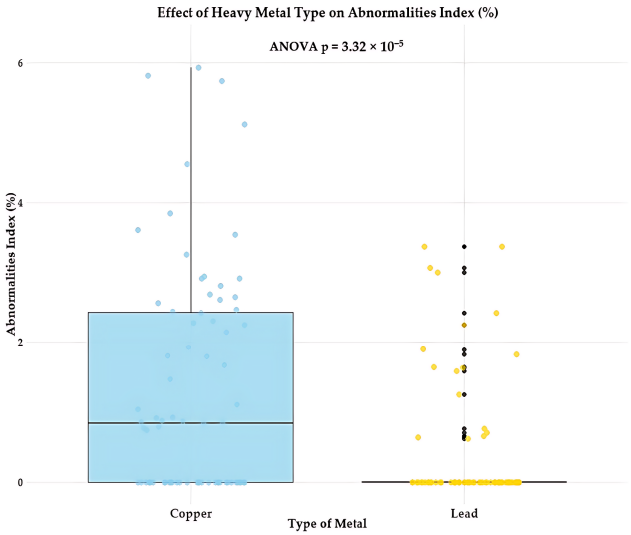

Figure 6. Boxplot showing the difference between the impact of Pb^{2+} and Cu^{2+} on the abnormalities index.

The results obtained confirm a strong relationship between the mitotic index (MI) and the abnormalities index (AI) (Figure 6). As the concentration of the metal treatment increases, significant changes are observed in both the AI and MI.

Mitotic disruptions are closely linked to, and often a result of, the stress caused by these metals, which induce chromosomal aberrations. Consequently, this leads to the inhibition of the mitotic index and therefore, the reduction in growth capacity (Figure 6).

Copper induces abnormalities, even at the initial concentrations of 0.25 mM, while maintaining mitotic activity relatively within normal limits. This highlights that the integrity of the cell division spindle is affected, but without completely halting the cell division capacity. Lead, on the other hand, shows a decrease in the MI at treatment concentrations, with more pronounced effects, along with an increase in the AI. Thus, both metals studied exhibit dose-dependent toxicity, with different toxicity thresholds, depending on the metal. The effects are visible for copper, even at concentrations as low as 0.25 mM, whereas for lead, they become more evident starting at 0.50 mM and above (Figure 6).

3.5. Correlations Between Cytological Indices Under Copper and Lead Treatments

Correlation analyses of cytological dynamics under the influence of different treatments have revealed varying correlation coefficients, depending on the treatment applied.

According to the results, Cu^{2+} has a more acute impact on cellular division, highlighted by more extreme correlations compared to those for Pb^{2+}.

Regarding the correlation coefficient between the mitotic index (MI) and the abnormality index (AI), the results indicate a negative correlation. This underlines that as the mitotic index decreases, the number of abnormal cells increases. Our results show a significantly stronger negative correlation for Cu^{2+} (−0.69) compared to Pb^{2+} (−0.29), further highlighting the higher genotoxic effect of Cu^{2+} (Figure 7).

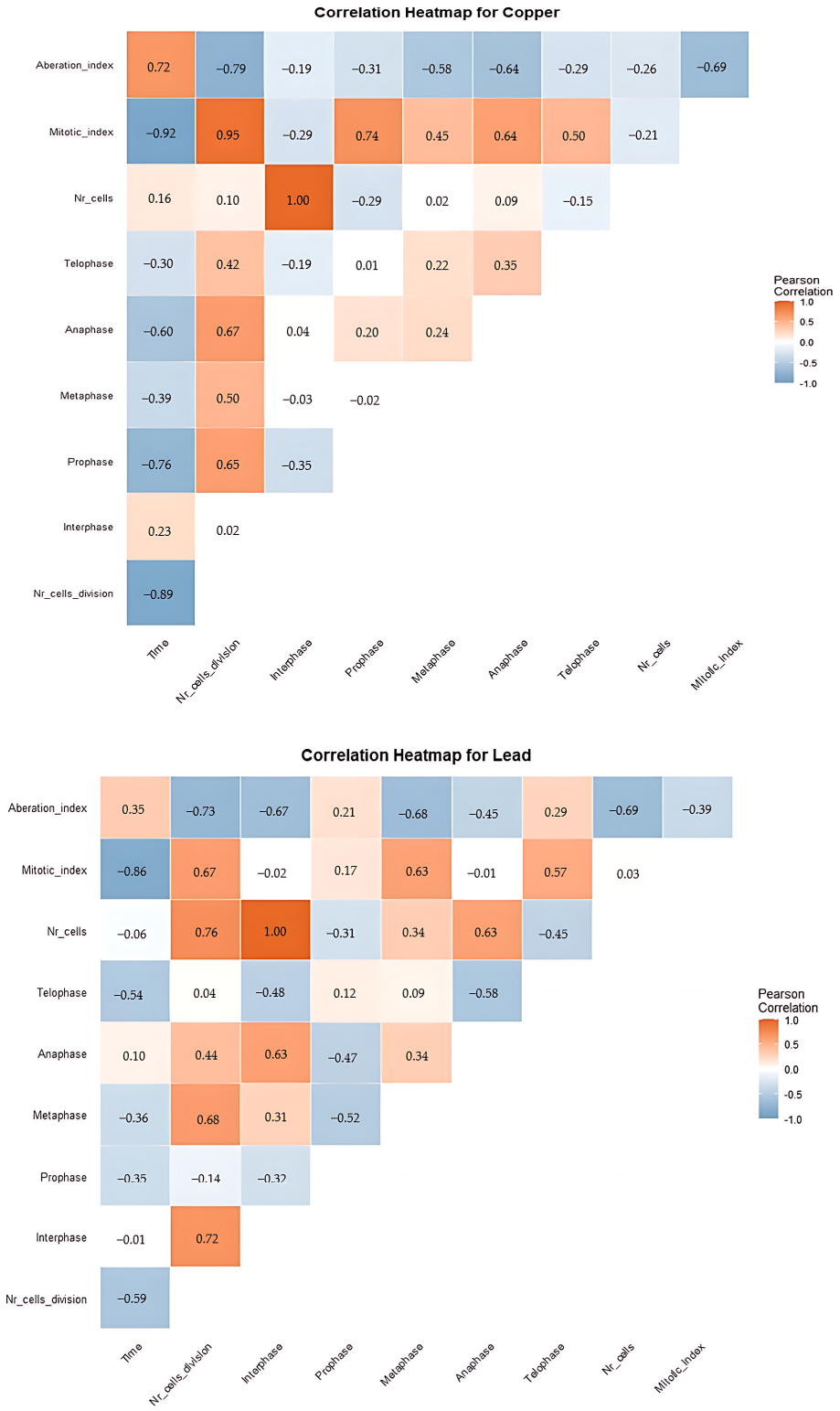

Figure 7. Mitotic index and abnormality index dynamics under Pb^{2+} vs. Cu^{2+} treatments.

Exposure time also shows a negative correlation during heavy metal treatments, with a correlation of −0.92 in the case of Cu^{2+} and −0.86 for Pb^{2+}. This indicates that the mitotic index decreases significantly for both metals as exposure time increases (Figure 7).

The number of abnormalities is also strongly linked to the number of dividing cells, showing a negative correlation for both metals studied. The greater accumulation effect in favor of Pb^{2+} can be observed in the correlation of −0.67 between the AI and cells in interphase. This suggests that as the treatment concentration increases, Pb^{2+} accumulates in the cells, halting and preventing cellular division. On the other hand, the same is not true for the correlation between the AI and the number of cells in interphase for copper, where the correlation is only −0.19. However, Cu^{2+} shows a stronger correlation with the mitotic index (−0.69). This indicates that copper's effect on halting cellular division is much more acute compared to lead. Lead, on the other hand, exhibits a cumulative effect, disrupting mitotic activity as it accumulates in the cells (Figure 7).

4. Discussion

Heavy metals are more persistently present in our lives and activities due to the high levels of industrialization [68,69], making it essential to better understand the maximum permissible limits [70]. Over time, their accumulation can ultimately trigger significant inhibition of the physiological activity of both plants and humans [71]. One of the major issues regarding heavy metals is the significant variation in regulations and the wide margin of error concerning the maximum allowable limits for heavy metals. It remains unclear at what precise concentration a heavy metal becomes toxic [33–35,72,73].

Cellular toxicity effects can be demonstrated by phenomena such as irreversible vacuolization, where the mitotic spindle becomes blocked and displaced to the outer edge of the vacuole, ultimately leading to the cessation of the cell division process. Irreversible vacuolization not only halts the process of cell division but is also associated with the phenomenon of programmed cell death (PCD) [74]. Irreversible vacuolization and dynamic vacuole formation in the process of toxicity are observed in several species, including *Allium cepa* [34,75].

The *Allium* test enabled the identification of genotoxic effects and the comparison of the toxicity levels of Cu^{+2} and Pb^{+2}. According to the experiment, which involved a direct proportional relationship between the Cu^{+2} and Pb^{+2} treatments, the genotoxic impact of concentration and exposure time were revealed. The findings revealed that exposure to increased doses of lead and copper significantly reduces the mitotic index (MI), while increasing the abnormalities index (AI). On the other hand, the ANOVA test evaluating dose and exposure time was significant only in the case of copper, reflecting that as the dose increases, the acute effect of copper becomes more pronounced. This was also observed in the correlation analyses.

The impact of copper is reflected in the observation that the MI was inhibited by 50% (IC_{50}) at lower concentrations, starting from a dose of 0.50 mM. Copper exhibited a stronger negative correlation between the mitotic index (MI) and the abnormalities index (AI) (−0.69), reflecting the genotoxicity that causes disruptions at the cellular level.

In contrast, for lead, the IC_{50} was observed at concentrations starting from 0.75 mM, exhibiting a negative correlation between the mitotic Index (MI) and the abnormalities index, which, at −0.29, was lower than that of copper. The cumulative effects of heavy metals, particularly lead, become evident, with a significant inhibition of cell division observed at high concentrations (1 mM) after 72 h.

In plants, PCD should be termed as apoptotic-like PCD (AL-PCD), as suggested by Reape et al., 2008 [34,76]. Studies demonstrate the relevance of genotoxicity and AL-PCD induction in essential biomarkers for stress in *Allium* species [76], suggesting that there are

some similarities between AL-PCD and apoptosis, although they are clearly different in each process. Specific AL-PCD processes, i.e., chromatin condensation, vacuolization, and DNA fragmentation, were detected [34,76].

However, the *Allium* test proved effective in detecting genotoxic effects and comparing the toxicity of copper and lead. Our correlation analyses results indicate a higher impact of copper on mitotic activity compared to lead. On the other hand, the limitations of the study did not clearly reflect the acute and chronic effect differences between lead and copper. Furthermore, according to studies in literature, hormesis can have beneficial stimulatory effects at low doses, promoting cellular repair and an increase in the mitotic index. Studies demonstrate the capacity of the *Allium* test to detect the hormesis effect in cellular division [77]. However, the concentrations used in this study (0.25–1.00 mM) did not reveal a cellular hormesis response to copper and lead.

In perspective, to address the limitations of the study, *Allium* test investigation requires long-term exposure at lower concentrations to provide additional insights into the more chronic effect of lead compared to that of copper, as well as to investigate the cellular hormesis response. Other points to be considered include the need for future studies regarding the vacuolization process, ROS production, and their effects on *Allium* test regarding heavy metal exposure.

5. Conclusions

Our study demonstrated the capacity of the *Allium* test for monitoring the mitotic index (MI) and the abnormalities index (AI) using concentrations of copper and lead varying between 0.25–1.00 mM. One explanation could be the physiological role of copper, which demonstrates a greater impact, as it is more cytologically intimate with cellular metabolism than is lead.

The experiment reflects a greater inhibitions effect of copper compared to that of lead. Copper markedly inhibits mitotic division, demonstrating a significant reduction in the MI, in which the IC_{50} was observed at 0.50 mM. In contrast, for lead, the IC_{50} was observed at concentrations starting from 0.75 mM. The cumulative effect of lead becomes evident, with a significant inhibition of cell division observed at high concentrations (1 mM) after 72 h of treatment. The results revealed that the AI increases as the MI decreases, a trend particularly evident in copper, where a negative correlation of −0.69 was observed between the MI and AI, reflecting the more acute impact of copper compared to that of lead.

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