

# *In vitro* analysis of thimerosal genotoxicity and cytotoxicity effect



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## ABSTRACT

**Background:** Thimerosal is an organomercury compound with high mercury content. Thimerosal is located in the market as an effective bacteriostatic in a series of pharmaceutical products, ophthalmic and nasal products, immunoglobulin preparations, and as a preservative in vaccines. **Aims and Objectives:** Since it is a compound with living content, this study aimed to examine the genotoxic and cytotoxic potential of thimerosal in human lymphocytes culture. **Materials and Methods:** We used chromosome aberration analysis and cytokinesis-block micronucleus cytome (CBMN-Cyt) assay to test its genotoxic and cytotoxic potential in human lymphocyte culture. **Results:** Results showed that the frequency of structural chromosome aberrations and CBMN-cyt assay was significantly increased in treated cultures (1 µg/ml and 0.5 µg/ml) compared to the negative control. **Conclusion:** Obtained results and statistical analysis show that thimerosal is genotoxic and cytotoxic in human lymphocytes in tested concentrations.

**Key words:** Chromosomal aberration; Cytokinesis-block micronucleus cytome assay; Thimerosal

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## INTRODUCTION

Thimerosal is an organomercury preservative that has been in use in the pharmaceutical industry since 1927. This preservative is specific for its high mercury content: it contains as much as 49.55% of mercury.<sup>1</sup> In aqueous solutions, it decomposes into ethyl-mercury (EtHg) in hydroxide and the sodium salt of thiosalicylic acid. For decades, thimerosal has been marketed as an effective bacteriostatic in some pharmaceutical products, including topical antiseptic solutions and ointments, nasal sprays, and preservatives in vaccines and other injectable biological products.<sup>2</sup>

As it is a compound with a proven toxic effect on human cells, its utilization today is significantly reduced. It is mainly limited to using multidose packages (bottles) of vaccines

to prevent their bacterial and fungal contamination during production, storage, and application. It is also used as a pathogen inactivation component in the vaccine production process. Thus, it can often be found in individually packaged doses of vaccines as a residue after the procedure.

Since thimerosal, as a preservative present in some vaccines, contains mercury as a known cytotoxic and genotoxicant, there is a need to consider this compound's potential cytotoxic and genotoxic effects.

Concerning this, one study using the so-called Allium test demonstrated that the indicators of cytotoxic activity of mercuric chloride are more intense in proportion to the applied concentration of this compound.<sup>3</sup> Another important mechanism of mercury genotoxicity is its ability to produce free radicals that can cause DNA

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damage.<sup>4</sup> Based on previous research on thimerosal and its importance in vaccines, this study aimed to examine the potential cytotoxic and genotoxic effects of specific thimerosal concentrations on human peripheral blood lymphocyte culture.<sup>5</sup>

## MATERIALS AND METHODS

### Tested substance

Thimerosal was dissolved in dH<sub>2</sub>O and added to cultures at the 25<sup>th</sup> h of cultivation to final concentrations of 0.5 µg/ml and 1 µg/ml. Negative controls with the equivalent volume of dH<sub>2</sub>O were set up as well.

### Lymphocyte culture

Human peripheral blood samples were taken from three voluntary donors for research purposes. These were male donors aged 20–25, with no previously known history of exposure to mutagens. Donors were informed and gave written consent to participate in the research.

Lymphocyte cultures were induced in 15-mL sterile plastic tubes with conical base (Isolab GmbH, Wertheim Germany), which contained 5 mL of PB-MAX Karyotyping Medium (GIBCO-Invitrogen, Carlsbad, CA, USA) and 400 µl of peripheral blood. Cultures were harvested after 72 h of lymphocyte cultivation at 37°C, using a standard procedure.<sup>6</sup>

### Chromosome aberration (CA) assay

For the CA analysis, lymphocytes were kept in metaphase by adding colcemid (Sigma-Aldrich, St.Luis, MO, USA) to the final 0.18 µg/ml concentration for 90 min before cell harvesting. Two hundred fifty metaphases were analyzed per treatment and controls. The analysis was performed on an Olympus BX53 electric light microscope under an immersion lens at a magnification of ×1000.<sup>7,8</sup> Structural chromosomal aberrations were scored and registered according to the International System for Human Cytogenetics Nomenclature.<sup>9</sup>

### Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

For the CBMN Cyt test, the cultivation of human peripheral blood was performed according to the Fenech protocol. The total duration of cultivation and incubation of human lymphocytes was 72 h.<sup>10</sup> Test substances were added 24 h after cultivation. Cell division was blocked at 45 h by adding 4.5 µg/ml cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA). Microscopic analysis was performed at ×60 magnification using an Olympus BX53 microscope. The frequency of micronuclei (MN), nuclear buds (NBs), and nucleoplasmic bridges (NPBs) was analyzed on at least 1000 binuclear lymphocytes.<sup>11,12</sup> The frequency

of mononuclear, binuclear, trinuclear, and tetranuclear lymphocytes, apoptosis, and necrosis was analyzed on at least 500 cells per sample.<sup>13</sup>

### Statistical analysis

ANOVA Two-Factor with Replication and ANOVA Single Factor (Microsoft Excel 2016 software) were used to determine a statistically significant difference between the arithmetic means of the observed cytotoxic and genotoxic parameters between controls and lymphocyte cultures treated with different thimerosal concentrations.

## RESULTS

Since many people are exposed to mercury and its derivatives, various animal and clinical studies have been done.<sup>14</sup> A review article by Sánchez-Alarcón et al., presented multiple studies of the genotoxicity of mercury and its derivatives *in vitro* and *in vivo* in human populations. The study found that peripheral blood cultures and, in some cases, oral mucosa epithelial cells were mainly used for genotoxic changes, such as CA and MN.<sup>15</sup> We were used only peripheral blood lymphocytes in our study.

Structural aberrations were analyzed in metaphases chromosomes and classified as chromatid-type (chtb) (breaks and minutes) and chromosome-type (chrb) aberrations (breaks, minutes, and rearrangements, for example., dicentrics). The most frequent structural aberrations in cultures treated with thimerosal were acentric fragments (Figure 1) and chromatid breaks (Figure 2). Endoreduplication has also been observed in cultures treated with 1 µg/ml thimerosal in medium (Figure 3).

For the determined results of all structural aberrations, the values of arithmetic means and standard deviation were calculated. In cultures of negative control,

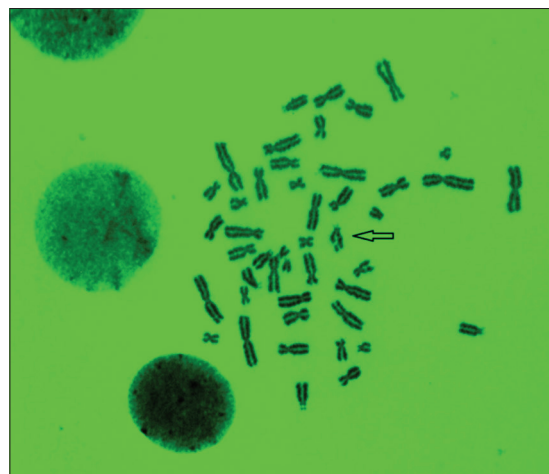
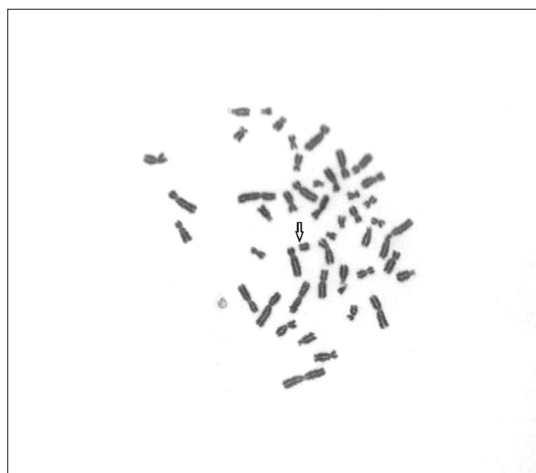
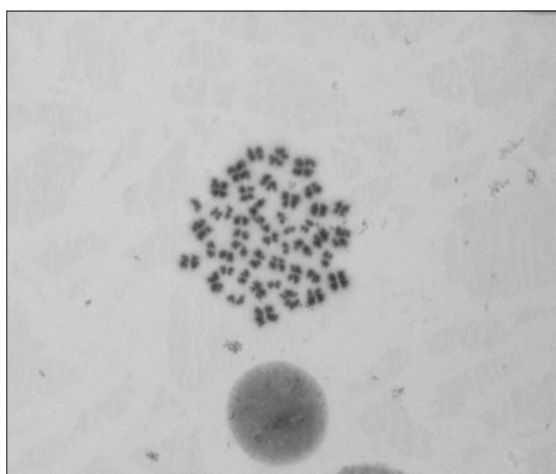


Figure 1: Chromatid break, thimerosal 1 µg/ml



**Figure 2:** Acentric fragments, thimerosal 1 µg/ml



**Figure 3:** Endoreduplication, thimerosal 1 µg/ml

one and no variation was recorded for different samples ( $X_{av} \pm s \rightarrow 1 \pm 0$ ). In cultures treated with 0.5 µg/ml thimerosal in medium, the arithmetic mean of total structural aberrations is  $6.67 \pm 3.06$ . Standard deviation, in this case, is significantly smaller in chromatid ones compared to chromosomal abnormalities. For cultures incubated in a medium with thimerosal at a concentration of 1 µg/ml, the arithmetic mean for total aberrations is  $9 \pm 1.73$ , the deviation is significantly smaller in chromatid aberrations. There was also a statistically significant difference between structural aberrations in thimerosal cultures and the negative control (Table 1).

The frequency of micronuclei in the negative control ranges from 3-4 micronuclei, with an arithmetic mean of  $3.67 \pm 0.71$ . After treatment of lymphocytes with 0.5 µg/ml thimerosal, the arithmetic mean is  $10 \pm 0$ . The frequency of the micronucleus, its arithmetic mean, was highest in the sample treated with a higher thimerosal concentration of 1 µg/ml and was  $15.57 \pm 1.53$ .

**Table 1: Presentation of the results of two-way ANOVA: comparison of aberrant changes in the tested cultures with the negative control**

ANOVA - Aberrations	P-value	F
Thimerosal 0.5 µg/ml and negative control	0.000427439	23.12
Thimerosal 1 µg/ml and negative control	6.77392E-08	135.5294

ANOVA: Analysis of variance

It can be concluded that an increase in the number of micronuclei was observed with an increase in the concentration of thimerosal (Figure 4).

By calculating the significance level by ANOVA test, a significant difference in the arithmetic means of the micronucleus frequency between the negative control and thimerosal treatment with 0.5 µg/ml and 1 µg/ml was determined. When using the micronucleus frequency between the negative control and the treated cultures, the culture treated with thimerosal at a 1 µg/ml- $p = 9.956 \times 10^{-6}$  had the lowest P-value, thus the difference of the highest significance. In contrast, this value for the culture treated with thimerosal concentration 0.5 µg/ml-  $p = 0.00126$ . Cytostatic and cytotoxic effects expressed as nuclear division indexes (NDI) and the cytotoxicity index of nuclear division (NDCI) were calculated after scoring mono-, bi-, tri-, and tri tetranuclear cells as well as apoptosis and necrosis in at least 500 cells per treatment.

Results of the analysis of micronuclei, nuclear buds, and nucleoplasmic bridges frequencies, nuclear division index, and nuclear division cytotoxicity index in human lymphocyte culture are presented in Table 2 as the means  $\pm$  standard deviation. It can be seen that the values of these parameters in terms of frequency increase/decrease in individual treatments and negative control are mutually monitored.

## DISCUSSION

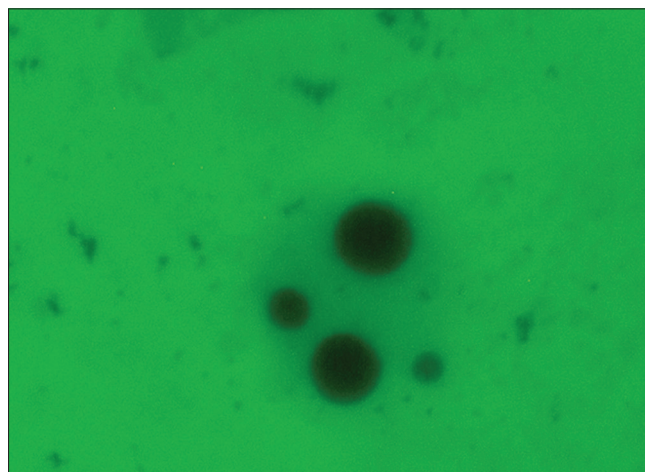
Since thimerosal is specific for its high mercury content: it contains as much as 49.55% of mercury, the results obtained in this study follow other works in which the cytotoxicity (increased number of chromosomal aberrations) of mercury was analyzed.<sup>16-18</sup>

In this study, a higher thimerosal concentration of 1 µg/ml was recorded as the highest intensity of chromosomal changes. In addition to analyzing structural CAs, one study showed that thimerosal caused toxic changes *in vitro*, including mitochondrial neuronal death, oxidative stress, and apoptosis of HeLa S cell line epithelial cells.<sup>19</sup>

**Table 2: CBMN-cyt assay in human lymphocytes treated with thimerosal**

Treatment	Micronuclei	Nuclear buds	Nucleoplasmic bridges	Nuclear division index	Nuclear division cytotoxicity index
Negative control	3.67±0.71	1.33±1.15	1.00±1.00	1.61±0.21	1.61±0.20
Thimerosal 0.5 µg/ml	10.00±1.20	2.00±1.73	2.00±1.00	1.34±0.11	1.36±0.13
Thimerosal 1 µg/ml	15.67±1.53	4.00±3.00	4.67±1.53	1.42±0.13	1.43±0.14

CBMN-cyt: Cytokinesis-block micronucleus cytome

**Figure 4:** Binuclear cell with two micronuclei

Similar results were recorded after performing cytokinesis-block micronucleus cytome assay in the domain of micronucleus frequency.<sup>20</sup> It can be concluded that an increase in the number of micronuclei was observed with an increase in the concentration of thimerosal (10.00±1.2 15.57±1.53). This result was expected, given that the number of structural chromosomal aberrations increased with increasing thimerosal concentration. When comparing the frequency of micronuclei between negative control and treated cultures, the culture with thimerosal with a concentration of 1 µg/ml  $P=9.956 \times 10^{-6}$  had the lowest P-value, and thus the difference of the highest significance, while culture treated with thimerosal concentration 0.5 µg/ml had the p-value of 0.00126. When analyzing the frequency of NBs and NPBs, it generally increases in the treated samples concerning the negative control culture.

In addition to the analysis and frequency of micronuclei, the frequency of apoptosis and necrosis was also analyzed. Determination of mitotic index and the frequencies of apoptosis and necrosis are essential parameters based on which the cytotoxic effects of the tested substances on lymphocyte culture are assessed.<sup>21</sup>

A sign of the growth of cytotoxic effects on lymphocyte culture is the increased frequency of apoptosis and necrosis. In comparison, a decrease in the mitotic index is a sign of cytotoxic effects on human peripheral blood lymphocytes. This study showed that the frequency of

apoptosis and necrosis was the same in cultures treated with thimerosal 0.5 µg/ml and 1 µg/ml. Based on their frequency and number of micronuclei, the cytotoxicity index of the nuclear division was calculated. When it comes to the values of the nucleus division index and the NDCI, the highest calculated values were recorded in control and the lowest in the culture treated with thimerosal 0.5 µg/ml.

Two-way ANOVA showed no significance between calculated values of NDI in treatments and negative control. No linear association between concentration and cytotoxicity indexes was observed.

In determining thimerosal concentrations in this study, research by Westphal et al., was analyzed, where the results of CBMC Cyt assay were used as a biomarker of genotoxicity. The research found that a significant increase in the appearance of micronuclei was seen at a thimerosal concentration of 0.05–0.5µg/ml, which was confirmed in 14–16 experiments. This study also found that toxicity and toxicity-related growth of micronuclei in human cells were recorded at concentrations of 0.6 µg/ml thimerosal and above.<sup>22</sup> These data served as a reference in selecting thimerosal concentrations for this study. Selected concentrations (0.5 µg/ml and 1 µg/ml) after CBMN cyt assay analysis of micronucleus frequency showed the existence of genotoxic effects to the negative culture and that several changes occurred related to the thimerosal concentration of 1 µg/ml.

Given that thimerosal is used as a preservative in vaccines, there are concerns about its cyto-genotoxic effects on the human body. For this reason, various regulatory bodies such as the Food and Drug Administration have for years encouraged the cessation of the use of thimerosal in multidose packages of vaccines and the greater use of individual packages in the so-called ready to use a form of vaccine where it is not necessary to add this preservative.<sup>23</sup>

Another reason for not using thimerosal is his alleged association with autism. There is numerous research on the subject. According to some research, a more significant amount can lead to autism, and for others, a particular thimerosal concentration is not related to autism.<sup>24-27</sup>

On the other hand, the Global Advisory Committee on Vaccine Safety considers that thimerosal as a preservative for inactivated vaccines should be continued. Because thimerosal enables millions of people worldwide with access to life-saving vaccines, no safer and equally effective alternative to many vaccines has been identified yet.<sup>28</sup> Testing and introducing a new preservative in multidose vaccine packaging is challenging for vaccine manufacturers. It is a lengthy process in terms of testing and approval by regulators with very high financial investment requirements yet with an uncertain outcome. A particular danger is that controversies over preservatives in vaccines lead to the fact that, unfortunately, a part of the population perceives vaccines as unsafe and unnecessary. However, vaccination is recognized as one of the most successful public health measures.<sup>29</sup>

### Limitations of the study

Future thimerosal studies should focus on assessing cytotoxic effects using more human lymphocyte samples and using additional cell models.

## CONCLUSION

The significance of this research is reflected in obtaining data on potential events at the cellular and molecular level after the administration of thimerosal found in vaccines. The results of this research can be the basis for further studies of cytotoxicity and genotoxicity of thimerosal and other substances from the environment that contain mercury in their composition, especially from the aspect of mutagenicity.

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**Author's Contributions:**

**MMĐ**- Concept and design of the study prepared the first draft of the manuscript, interpreted the results; **AA**- Reviewed the literature and manuscript preparation; **DR**- Concept, coordination, statistical analysis; **IA**- Preparation of manuscript, and revision of the manuscript.

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