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Original Research Article

Genotoxic and cytotoxic effects of thiomersal and paracetamol combination

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ABSTRACT

Background: Thiomersal is used as a preservative of some vaccines or as a trace from the pathogen inactivation process in vaccine production. Prophylactic use of paracetamol upon vaccination is still common, even though paracetamol decreases immune response on some vaccines. Considering the cytotoxic and genotoxic potential of thiomersal and paracetamol and possible interaction in vivo, in vitro study was performed.
Methods: The genotoxic and cytotoxic effects of thiomersal and paracetamol combination were examined on human lymphocyte cultures by using two methods: analysis of chromosomal aberrations and cytokinesis-block micronucleus cytome assay. Blood samples of three healthy donors were analyzed with the following concentrations of tested substances: thiomersal 1 µg/ml and 0.5 µg/ml, paracetamol 20 µg/ml, thiomersal 0.5 µg/ml with paracetamol 20 µg/ml and thiomersal 1.0 µg/ml with paracetamol 20 µg/ml.
Results: The analysis of structural chromosomal aberrations was significantly increased in all treated cultures. In cells treated with the combination of thiomersal 1 µg/ml and 20 µg/ml of paracetamol, the number of aberrations was significantly decreased. Cytokinesis-block micronucleus cytome assay analyses showed significantly increased micronucleus frequency in lymphocytes cultivated with thiomersal 1 µg/ml compared to lymphocytes cultures exposed to thiomersal 0.5 µg/ml.
Conclusions: Induction of structural chromosome aberrations and micronucleus is shown as a sign of genotoxicity for the examined concentrations of thiomersal and paracetamol. The suppressing effect of paracetamol on thiomersal genotoxicity in lymphocytes culture treated with thiomersal was shown to be indicative of further examination of paracetamol use in the prevention of genotoxicity.

Keywords: Thiomersal, Paracetamol, Aberrations, Micronucleus

INTRODUCTION

Thiomersal is an organomercury preservative that contains as much as 49.55% mercury. In aqueous solutions, it decomposes into ethylmercury (EtHg) in the form of hydroxide and the sodium salt of thiosalicylic acid.[1] Although its use has been reduced, thiomersal is still present as a preservative in multidose containers of some vaccines or as a trace from the pathogen inactivation process in vaccine production.[2] The link between thiomersal in vaccines and autism has been widely investigated. However, food and drug administration

(FDA) reported no evidence of harm from using this preservative.[3] Paracetamol, a widely used analgesic and antipyretic, is most often used to treat postimmunization pyrexia. Prophylactic use of paracetamol upon vaccination is still present, even though in these cases, paracetamol decreases the immune response to some vaccines. Paracetamol increases the frequency of chromosomal damage in mammalian cell lines, isolated human lymphocytes, and experimental animal studies.[4] Two independent studies showed increased chromosomal damage in human lymphocytes after taking therapeutic doses of paracetamol, while a third study gave negative results. The inhibition of ribonucleotide reductase probably causes paracetamol-induced chromosomal damage, indicating that a given dose of paracetamol may induce a threshold level for chromosomal damage. Because of the interaction possibility of these two substances in vivo, it was found attractive to examine their genotoxicity in vitro. Considering the described elements of cytotoxicity and genotoxicity of thiomersal and paracetamol, this study aimed to evaluate the possible influence of paracetamol on the intensity of the cytotoxic and genotoxic effects of the investigated concentrations of thiomersal.

METHODS

In this study, the investigation of cytotoxicity and genotoxicity of thiomersal and paracetamol combinations, was performed on peripheral blood lymphocytes by using the method of analysis of chromosomal aberrations and cytokinesis-block micronucleus cytome (CBMN-cyt) assay. Research was performed in Center for Genetics at Medical Faculty University of Sarajevo (May-2019 to Oct-2019). Cultivation of human peripheral blood takes place in two separate processes: the Moorhead method for testing chromosomal aberrations and the Fenech method for the CBMN-cyt assay. The test substances, thiomersal and paracetamol, are added to the cultures 24 hours after cultivation, and the cultivation lasts a total of 72 hours. The statistical analysis of the obtained results and the determination of the statistical difference between the negative control and the cultures treated with the tested substances, as well as the determination of the influence of paracetamol on the potential cytotoxicity and genotoxicity of thiomersal.

Blood sampling

Human peripheral blood samples for research purposes were taken from 3 voluntary healthy donors, men aged 20-25 years, nonsmokers, with no previous known history of exposure to mutagens (e.g., chemicals, ionizing radiation). Donors were informed and gave their written consent to participate in the research. Human blood is collected by venipuncture in vacutainers containing lithium heparin, the so-called green tubes (Vacutainer Systems, Plymouth, UK). The volume of blood taken is 4 ml (2 vacutainers each), and the blood is taken from the donor on the day of the test. It is transported to the laboratory for testing in a short period under controlled temperature conditions of +4°C.

Tested substances

The following substances were used in the research: Thiomersal (thiomersal), sodium ethyl (2-sulfanylbentoato (2)-O, S) mercurate (1-) (Sigma-Aldrich, St. Louis, MO, USA), and pure paracetamol in the form of the manufacturer's reference standard (Huzhou Konch Pharmaceutical Co., China). Thiomersal and paracetamol were added to the culture at the 24th hour of the cultivation in final concentrations (thiomersal 0.5 µg/ml, 1 µg/ml, paracetamol 20 µg/ml, and combinations of thiomersal 0.5 µg/ml+paracetamol 20 µg/ml and thiomersal 1 µg/ml+paracetamol 20 µg/ml). In parallel with the tested substances, a negative control was set using distilled water in a volume identical to that used for the solutions of the tested substances.

Lymphocyte culture

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 hours of lymphocyte cultivation at 37°C, using a standard procedure (hypotonic treatment with 0.075M KCl, triple ethanol-acetic acid fixation, followed by microscope slide preparations and staining in 5% Giemsa).[5] Cultivation was carried out under controlled temperature and CO_2 content conditions in an incubator (at 37°C and with a gas ratio of 5% CO_2 and 95% atmospheric air).

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 control. The analysis was performed on an Olympus BX53 (Tokyo, Japan) light microscope under an immersion lens at a magnification of ×100.[6] Structural chromosomal aberrations were scored and registered according to the International system for human cytogenetics nomenclature.[7]

Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The analysis of genotoxic and cytotoxic of thiomersal and paracetamol were evaluated in human lymphocyte cultures by applying cytokinesis-block micronucleus cytome assay in vitro. To block cytokinesis, 4.5 µg/ml of cytochalasin B (Sigma-Aldrich, St. Louis, MO, USA) is added to the culture at the 44th hour from the start of cultivation. Microscopic analysis was performed at ×60 magnification using an Olympus BX53 microscope (Tokyo, Japan). The

analysis of preparations obtained after conducting the CBMN-cyt test includes the identification of micronuclei (MN), nuclear buds (NBs), nucleoplasmic bridges (NPBs), mono-, bi-, tri- and tetranuclear cells, apoptosis, and necrosis. The frequency of MN, NBs, and NPBs was analyzed on at least 1000 binuclear lymphocytes.[8] The frequency of mononuclear, binuclear, trinuclear, and tetranuclear lymphocytes, apoptosis, and necrosis was analyzed on at least 500 cells per sample.[9]

Statistical analysis

The data, obtained after microscopic analysis of cultures exposed to the action of the tested substances, were statistically processed using adequate statistical and mathematical functions from the Microsoft Excel 2016 software.

Arithmetic means (Xav) and standard deviations (s) were calculated as a measure of variability. Data related to structural aberrations (chromosomal and chromatid), mitotic indices (viability based on NDI and NDCI values), and CBMN-cyt test results (number of micronuclei, nuclear buds, and nucleoplasmic bridges) were processed by statistical analysis. The statistical values were determined for each tested cell line, including the negative control. To determine a statistically significant difference between the arithmetic means of the observed cytotoxic and genotoxic parameters between control and lymphocyte cultures treated with tested combinations of substances, analysis of variance (ANOVA) was used as ANOVA two-factor with replication and ANOVA single factor.

RESULTS

Analysis of structural aberrations of chromosomes

We analyzed 200 metaphase cells in each of the 6 lymphocyte cultures for all three samples/donors separately (18 lymphocyte cultures in total). In a culture of lymphocytes incubated in a medium with 20 µg/ml paracetamol and 1 µg/ml thiomersal, a dicentric with two acentric fragments was found only in one donor (Figure 1).

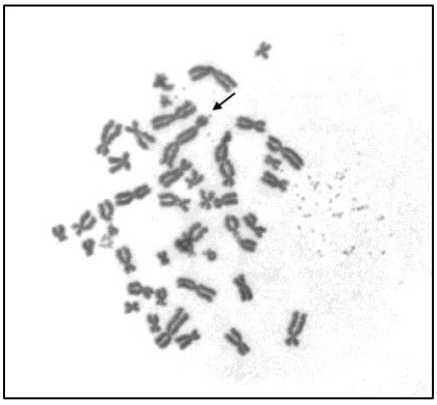

Figure 1: Dicentric chromosome.

Arithmetic means and standard deviation values were calculated for the established results. The arithmetic mean of all structural aberrations (chromatid and chromosomal) in negative control cultures is 1, and no deviation was recorded for different samples (Xav±s→1±0). For thiomersal concentration 0.5 µg/ml with 20 µg/ml paracetamol, the result is 4.67±2.52, and for thiomersal concentration 1 µg/ml with 20 µg/ml paracetamol, 5.33±0.58. The most frequently recorded of all aberrations was the appearance of an acentric fragment (ace) as a chromosomal type aberration; a total of 29. In the negative control, there were no such aberrations. In contrast, in the culture treated with thiomersal 1 µg/ml and the culture treated with paracetamol 20 µg/m, their maximum number of 4 observed aberrations occurred on 200 examined metaphase cells. In treated lymphocyte cultures, after acentric fragments, the next most frequent occurrence is the appearance of chromatid minute fragments; a total of 27. They are followed by the appearance of chromatid breaks; a total of 21.

Results of the CBMN-cyt test

The frequency of micronuclei (MN), nuclear buds (NP), and nucleoplasmic bridges (NPM) (Figure 2) was analyzed in at least 1000 binuclear lymphocytes (BN). The frequency of mononuclear, binuclear (Figure 3), trinuclear, tetranuclear lymphocytes, apoptosis, and necrosis was analyzed on at least 500 cells per sample.

It can be concluded that an increase in the number of micronuclei was observed with increasing thiomersal concentration (10±0→15.57±1.53). Also, compared to the negative control, slightly lower growth in the average value of the frequency of micronuclei was recorded in the sample with the addition of paracetamol alone (6.33±1.41) with significant differences in the number of micronuclei for different samples (3-11 micronuclei).

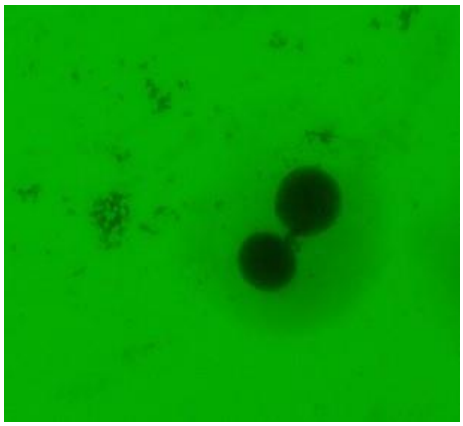

Figure 2: Cell with nuclear bridge.

The absolute frequency of micronuclei increases with the increase in the concentration of thiomersal added to paracetamol from 0.5 µg/ml to 1 µg/ml (10.33±0.58→11±0.71). By calculating the level of

significance through a one-way ANOVA test, a significant difference in the arithmetic means of the frequency of micronuclei was determined between the negative control and the treatments: thiomersal 0.5 µg/ml, thiomersal 1 µg/ml, thiomersal 0.5 µg/ml and paracetamol 20 µg/ml, and thiomersal 1 µg/ml and paracetamol 20 µg/ml.

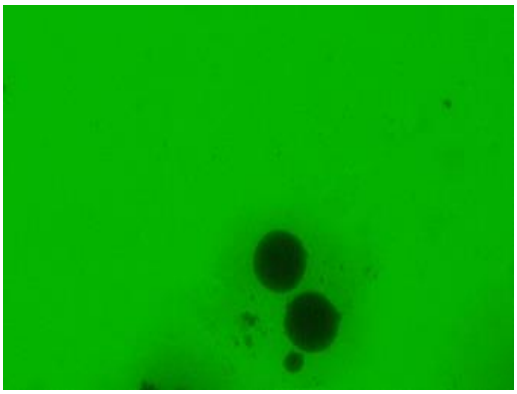

Figure 3: Binucleated cell with micronuclei.

Only this difference was not found to be significant for the frequency of micronuclei in the culture with the treatment of 20 µg/ml paracetamol and the negative control. When comparing the frequency of micronuclei between the negative control and treated cultures, the culture treated with thiomersal at a concentration of 1 µg/ml had the lowest p value, and thus the difference of the highest significance; p=9.956×10-6, while this value for the culture treated with thiomersal concentration 0.5 µg/ml p=0.00126. There is an evident and statistically significant difference between the frequency of micronuclei in cultures with the addition of thiomersal compared to cultures treated with a combination of thiomersal and paracetamol. The frequency of micronuclei in cultures treated with thiomersal 0.5 µg/ml and paracetamol 20 µg/ml compared to cultures treated only with thiomersal 0.5 µg/ml was found to be significantly different, i.e., smaller (p=0.000226). A statistically significant difference is even more pronounced when it comes to the frequency of micronuclei in cultures treated with thiomersal 1 µg/ml and paracetamol 20 µg/ml compared to cultures treated only with thiomersal 1 µg/ml ($p=1.58\times10^{-5}$). The difference in the frequency of micronuclei in cultures treated with thiomersal 0.5 µg/ml and thiomersal 1 µg/ml was also significant.

DISCUSSION

The genotoxic potential of chemical substances was determined by analyzing structural aberrations of chromosomes on human peripheral blood lymphocytes under in vitro conditions. It was found that both investigated concentrations of thiomersal and paracetamol, both separately and together, led to significant differences in the manifestation of effects on the structure of chromosomes compared to the negative control. A higher thiomersal concentration of 1 µg/ml was recorded as the concentration with the highest intensity of chromosomal changes. At the same time, the presence of paracetamol proved to reduce its effects on hereditary material. On the other hand, the appearance of dicentrics with two acentric fragments (donor 1) was observed in the lymphocyte culture incubated in the medium with 20 µg/ml paracetamol and 1 µg/ml. However, since this is an isolated case, we cannot discuss the significance of the more intense genotoxic effect of the combination of these substances on the tested cultures. Sánchez-Alarcón et al used mainly peripheral blood cultures and, in some cases, oral mucosa epithelial cells to identify genotoxic alterations in populations chronically exposed to mercury levels below the safety values defined by the WHO.[10] Identical changes were recorded after performing the cytokinesis-block micronucleus test in the micronucleus frequency domain. During this test, a significant suppressive effect of paracetamol on the manifestation of the genotoxic effects of thiomersal in the hereditary material was also recorded at both tested concentrations of thiomersal: 0.5 µg/ml and 1 µg/ml. In this study, the higher tested concentration of thiomersal also leads to significantly more significant genotoxic effects on the tested lymphocyte culture.

During the cytotoxicity test of the combination of thiomersal and paracetamol, the observed cytostatic and cytotoxic effects were not statistically significant in any of the lymphocyte cultures tested. Previous research has not confirmed the genotoxic effect of paracetamol in therapeutic doses. In contrast, at higher concentrations (> 200 µg/ml), the genotoxic impact, as well as its antiproliferative activity, have been proven.[4,11,12] Reviewing the results of some research on the cytotoxicity and genotoxicity of paracetamol, the diversity of the research results, including the differences in the in vivo and in vitro behavior of paracetamol, leads to the conclusion that there is no clear or established way of its behavior as a cytotoxic and genotoxic.

The use of thiomersal in the pharmaceutical industry cannot and will not be stopped immediately and thoroughly. This would disrupt the supply of vaccines necessary to maintain the public health segment, especially in countries with weaker economic power that can provide immunization programs exclusively by using multidose vaccine packages.[1] On the other hand, in the USA, children six years of age and younger are routinely immunized with vaccines that do not contain thiomersal.[3] The importance of this research is reflected in obtaining data on potential events at the cellular and molecular level after the application of thiomersal, found in vaccines. Considering its long half-life in the human body and the fact that, on the other hand, paracetamol is the drug of choice in the treatment of post-immunization pyrexia, especially in children, the commutagenic effect of these substances is possible in vivo, especially at the site of vaccine administration. The possibility of joint action of these substances is all the greater because the use of paracetamol in the prophylaxis of post-immunization side effects

(treatment of pain and elevated temperature) is still present in practice, regardless of the valid recommendations on reducing the immunogenicity of the vaccine by the action of paracetamol. The suppressive effect of paracetamol on the manifestation of the genotoxic effects of thiomersal, determined by the analysis of genotoxicity markers, proved to be an essential result of this research. Considering cytotoxic and genotoxic potentials of both tested substances, diametrically opposite results were expected. The fact that the suppressive effect of paracetamol was not recorded as significant after the analysis of chromosomal structural aberrations in the combined treatment of lymphocytes with paracetamol and thiomersal of a lower concentration can be explained by the fact that the stated concentration of thiomersal generally does not cause a significant increase in the frequency of genotoxicity markers, as is the case with its higher concentration. Earlier studies indicated that the effects of thiomersal genotoxicity were significant only above a concentration of 0.6 µg/ml.[13]

Limitations

Limitation of current study was small sample size. It is necessary to conduct research on a larger sample in order to obtain even more accurate results.

CONCLUSION

Induction of structural chromosome aberrations and micronucleus is shown as a sign of genotoxicity for an examined concentration of thiomersal and paracetamol, and their standard treatment of lymphocytes cultures, except for paracetamol treated culture regarding micronucleus frequency. The suppressing effect of paracetamol on thiomersal genotoxicity in lymphocytes culture treated with thiomersal was shown as indicative for further examination of paracetamol use in the prevention of genotoxicity. The results of this research can be the basis for further investigations of the cytotoxicity and genotoxicity of thiomersal, as well as other substances from the living environment that contain mercury in their composition, especially from the aspect of commutagenity. It is necessary to conduct research on a larger sample in order to obtain even more accurate results.

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