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Genotoxicity of thimerosal in cultured human lymphocytes with and without metabolic activation sister chromatid exchange analysis proliferation index and mitotic index

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Abstract

Thimerosal is an antiseptic containing 49.5% of ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. In this study, we evaluated the genotoxic effect of thimerosal in cultured human peripheral blood lymphocytes using sister chromatid exchange analysis in culture conditions with and without S9 metabolic activation. This study is the first report investigating the genotoxic effects of thimerosal in cultured human peripheral blood lymphocyte cells using sister chromatid exchange analysis. An analysis of variance test (ANOVA) was performed to evaluate the results. Significant induction of sister chromatid exchanges was seen at concentrations between 0.2 and 0.6 μg/ml of thimerosal compared with negative control. A significant decrease ($p < 0.001$) in mitotic index (MI) and proliferation ındex (PRI) as well as an increase in SCE frequency ($p < 0.001$) was observed compared with control cultures. Our results indicate the genotoxic and cytotoxic effect of TH in cultured human peripheral blood lymphocytes at tested doses in cultures with/without S9 fraction.

Keywords: Genotoxicity; Thimerosal; S9 activation system; Mitotic index; Proliferation index

1. Introduction

Thimerosal (TH, sodium ethylmercury-thiosalicylate,) was developed by Eli Lilly in the 1930 s as an effective bacteriostatic, fungistatic preservative and has been widely used in multidose vials of vaccines and in ophthalmic, otic, nasal, and topical products. (Ball et al., 2001). TH contains 49.6% mercury by weight and releases ethylmercury as a metabolite. In the body, ethylmercury can be converted to inorganic mercury. Inorganic mercury is known to induce membrane and DNA damage (Ferrat et al., 2002; Ben-Ozer et al., 2000), and in cell culture conditions it was shown to be mutagenic and generate DNA breaks. Due to possible adverse health effects, investigations on its metabolism and toxicity are urgently needed. An *in vivo* study on chronic toxicity of TH in rats was inconclusive and reports on genotoxic effects in various *in vitro* and/or *in vivo* systems were contradictory. Little is known about the reactions of human peripheral blood lymphocyte at low concentrations, which can occur after using TH containing products.

In addition, there were reports on genotoxic effects of TH *in vivo*. A weak but significant increase in micronuclei and chromosome aberrations was seen in male Swiss CD–1 mice at doses between 10 and 20 mg/kg (Marrazzini et al., 1994); another study in used male and female (102/E1·C3H/E1) F1 mice and Swiss albino mice reported negative results (Adler et al., 1991).

Possible carcinogenic effects were investigated in one study on the chronic toxicity of TH in Fischer 344 rats (Mason et al., 1971). However, this study does not meet

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the requirements of the current guidelines and does not rule out a possible carcinogenic effect of TH.

In standard *in vitro* genotoxicity testing, an activation system is included with the purpose of generating electrophilic metabolites that can react with macromolecules including nucleic acids. Since 1970, this has been a hallmark of the Ames bacterial mutagenicity test, and its utility was apparent when some of the first environmental mutagens were characterized in this system. It was a natural extension of the pioneering work of the Millers who demonstrated bioactivation of xenobiotics to reactive metabolites (Miller, 1970). Today, S9 mix activation system has been applied in many *in vitro* cell cultures by many researchers (González Borroto et al., 2002; Marques et al., 2002). Many mutagens require oxidative metabolism to reactive species before demonstrating mutagenicity.

Genotoxic agents have the potential to interact with DNA and may cause DNA damage. SCE occurs spontaneously in proliferating cells and is regarded as a manifestation of damage to the genome. It has been commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure, and dose–response relationships for different chemicals have been reported in both *in vivo* and *in vitro* studies (Bal et al., 1998).

Genotoxic potential of chemical or different chemicals can be assessed with the sister chromatid Exchange (SCE) test that is highly sensitive (Aydemir et al., 2005; Çelik and Akbaş, 2005; Krause et al., 2003; López-Nigro et al., 2003; Tucker and Preston, 1996). SCE test uses genetic damage induced in cells by a compound as an indicator of its potential for mutagenicity. SCE test involves the exchange of DNA segments between two sister chromatids in a chromosome during cell replication. An increased frequency of SCE in the presence of the compound indicates its mutagenicity (Hoerauf et al., 1999; Holz et al., 1995). SCE has been widely used to determine cytogenetic response to chemical exposure, and dose–response relationships have been established in many *in vivo* and *in vitro* studies (Aydemir et al., 2005; Çelik and Akbaş, 2005; López-Nigro et al., 2003). The response of the SCE test is typically assessed in comparison with a positive control.

The purpose of this study was to determine the genotoxic sensitivity of peripheral blood lymphocytes to TH cytotoxicity and mutagenicity under with and without S9 fraction *in vitro*.

2. Materials and methods

2.1. Donors and collection of blood samples

The study was carried out by using blood samples from three healthy, non-smoking male donors, aged 20, 21 and 23 years. Approximately, 10 ml of blood was collected, by venipuncture, into syringes containing sodium heparin as anticoagulant. Blood was taken the same day of the initiation of the experiment between 9.00 and 9.30 a.m. to minimize possible confounding effects of dietary factors.

2.2. Test chemicals

Thimerosal (CAS No. 54–64–8) was obtained from Sigma (Fig. 1). Mitomycin C (MMC, CAS No: 50-07-7, Kyowa). Methanol, acetic acid, potassium chloride, sodium chloride, tri-sodium citrate-2-hydrate, di-sodium hydrogen phosphate-2-hydrate, potassium dihydrogen phosphate and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine was purchased from Fluka (Buchs, Switzerland). Phytohaemagglutinin (PHA, M form) was obtained from Gibco BRL Life Technologies (Paisley, UK) and prepared according to supplier's instructions. Heparin was purchased from Braun (Melsungen, Germany). 5-Bromo-2-deoxyuridine (BrdU) was obtained from Sigma. In the cultures without metabolic activation, the positive control was MMC at 2 μg/ml for treatments. For cultures with metabolic activation, cyclophosphamide (CP, CAS No. 6055-19-2, Sigma) was used as positive control at 1.4 μg/ml for testing SCE.

2.3. Dose selection

The dose range was selected according to concentrations (30–100 μg/ml) in vaccines of TH (Ball et al., 2001). TH was used at final concentration of 0.2, 0.4 and 0.6 μg/ml. In this study, "the highest dose of TH" 0.6 μg/ml was used as 1/50 of concentration in vaccines (30 μg/ml).

2.4. Metabolic activation

S9 from (Cat No: 452591) "BD Biosciences" was used as metabolic activation system. The rats used to produce the S9 were not given arochlor, phenobarbitol, or other chemicals. The S9 mix freshly prepared, consisted of 1 ml of S9, 0.33 ml of 1 M KCl, 0.32 ml of 0.25 M MgCl2·6 H2O, 0.25 ml of 0.2 M glucose-6-phosphate, 1 ml of 0.04 M NADP, 2.10 ml of distilled water and 5 ml of phosphate buffer (pH 7.4).

2.5. Genotoxic evaluation

2.5.1. Lymphocyte cultures and sister chromatid exchange analysis in peripheral blood lymphocytes

The study was carried out by using blood samples from three healthy non-smoking male donors. In three donors, results of clinical routine laboratory analyses were in normal range, and the absence of exposure to known genotoxicants was considered. The dose range was selected according to concentrations in vaccines of TH.

Lymphocyte cultures were prepared according to the technique of Moorhead et al. (1960) with slight modifications. Heparinized whole-blood (0.9 ml) was added to 5 ml of culture medium F10 (Gibco, USA), supplemented with 20% of fetal calf serum (Gibco, USA), 0.1% ml phyto-

hemagglutinin (Gibco), and antibiotics (10,000 IU/ml penicillin and 10,000 IU/ml streptomycin). 5-Bromo-2-deoxyuridine (9 μg/ml, BrdU, Sigma) was added to cultures at the beginning of the 72-h incubation period at 37 °C for SCE analysis. Lymphocytes were cultured in the dark for 72 h, and metaphases were blocked during the last 1.5 h with colcemid at final concentration of 0.2 μg/ml. Mitomycin C (2 μg/ml) was used as positive control. The cells were harvested by replacing the culture medium with KCl (0.075 M) in which cells were incubated for 20 min at 37 °C. The cells were fixed in Carnoy's fixative (methanol: acetic acid, 3:1 v:v) five times, and slides were kept at room temperature overnight. Air-dried slides were stained according to fluoresence-plus Giemsa method by Perry and Wolf (1974) with slight modification. The number of SCEs was counted in 50 second-metaphase cells from each of the duplicate cultures on coded slides. Thus, 100 cells were scored in blind per dose for SCEs.

In general, the same procedures were used for the assays conducted with and without metabolic activation. Nevertheless, in the case of cultures with metabolic activation, 24 h after the initiation of cultures, 0.5 ml of S9 mix were added together with the test agent (0.2, 0.4, 0.6 μg/ml). After an incubation period of 3 h at 37 °C, the test chemical and S9 mix were removed from the culture. Concurrent cultures, treated for 3 h without the activating system, were also set up. The pellet of lymphocytes was washed twice with 5 ml of RPMI 1640 medium and resuspended in complete medium and, after that, the cultures were incubated until the entire period of 72 h at 37 °C.

2.6. Cell proliferation kinetic (CPK) and mitotic index

The mitotic index (MI) was calculated as the proportion of metaphases among the total cell population by counting a total of 1000 cells. The cell proliferation kinetics was defined as the proportion of the relative frequency of first–division metaphases (M1, identifiable by uniform staining of both the sister chromatids), second division metaphases (M2, identifiable by differential staining of the sister chromatids), and third and subsequent division metaphases (M3, identifiable by nonuniform pattern of staining). Proliferation index (PRI) was calculated according to Ivett and Tice (1982). PRI is the average number of replications completed by metaphase cells and is calculated as follows:

$$\mathrm{RI} = 1 \times (\%\ \text{first division metaphases}) + 2 \times (\%\ \text{second division metaphases}) + 3 \times (\%\ \text{subsequent division metaphases})/\mathrm{N}$$

2.7. Microscopic observation

All slides were coded prior to scoring. SCE scoring was carried out by only one person (D.EKE) using a MİCROS MC300A light microscope at 1000× magnification under oil immersion. For the SCE analysis, 50 metaphases of second division were examined for each treatment and donor. A further 100 metaphases for each concentration were also scored to evaluate the proportion of cells that had undergone one to three divisions.

2.8. Statistical analysis

For the statistical analysis of SCE, the data were transformed using the square roots of the original values plus 1 ($\sqrt{(x+1)}$), this transformation is effective in stabilizing the dispersion and makes the variances independent of the means. Then, a one-way ANOVA was performed. The comparisons between groups were made using a post-hoc test, LSD (least significant difference) test. Statistical decisions were made with a significance level of 0.05.

3. Results

The results of the frequency of SCEs the values RI and MI for each dose and control groups are depicted in Tables 1 and 2, respectively. TH induced a dose–related increase in the frequency of SCE and decrease in the frequency of MI and PRI.

Figs. 2 and 3 show the dose–response characteristics of the cultures of human peripheral blood lymphocyte for SCE frequency SCE points, respectively. In lymphocyte cultures, a progressive increase in mutagenicity (increase in SCE frequency) was observed when TH dose was increased from 0.2 μg/ml to 0.4 μg/ml and 0.6 μg/ml under culture conditions with S9 and without S9 fraction. There is a significant difference between negative control and all dose of TH. It is found that significant difference is $p < 0.01$ for 0.2 μg/ml, 0.2 μg/ml + S9 and 0.4 μg/ml and 0.4 μg/ml + S9 but for 0.6 μg/ml and 0.6 μg/ml + S9 is $p < 0.001$ for SCE. No significant difference is found between 0.4 μg/ml and 0.4 μg/ml + S9 ($p > 0.05$).

In the lymphocyte cultures, decrease in the frequency of mitosis was detected for treatment with TH under our experimental conditions. A dose-dependent decrease was observed in the mitotic index. A significant difference was found between the doses of TH (0.2, 0.4 and 0.6 μg/mL,) under conditions with S9 and without S9 fraction and negative control ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Fig. 4 represents the decrease in mitotic index.

With respect to the cytostatic effects of the test compound, measured as a cell cycle delay (PRI), a reduction in cell proliferation was observed in three donors. In PRI

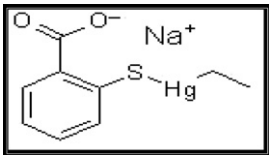

Fig. 1. Chemical structure of thimerosal.

Table 1
The frequencies of SCE and the PRI values in human lymphocyte treated with thimerosal (TH) in cultures with and without S9 *in vitro*

Treatments	Donor A		Donor B		Donor C		Mean ± SD	Mean ± SD
	SCE/M	PRI	SCE/M	PRI	SCE/M	PRI	SCE/M	PRI
NC	3.08	2.09	3.14	2.07	3.08	2.09	3.10 ± 0.03	2.08 ± 0.01
NC + S9	4.00	2.03	4.10	2.04	3.90	2.07	4.00 ± 0.10	2.04 ± 0.02
TH 0.2 μg/ml	5.20	2.00	5.20	2.00	5.40	2.01	5.26 ± 0.11**	2.00 ± 0.05***
0.2 μg/ml + S9	6.10	2.00	6.00	1.95	5.90	2.00	6.00 ± 0.10**	1.98 ± 0.02***
0.4 μg/ml	7.10	1.83	6.90	1.84	7.30	1.85	7.10 ± 0.20**	1.84 ± 0.01***
0.4 μg/ml + S9	7.30	1.80	7.30	1.81	7.20	1.83	7.27 ± 0.18**	1.81 ± 0.01***
0.6 μg/ml	9.90	1.74	10.10	1.73	10.20	1.75	10.06 ± 0.15***	1.74 ± 0.01***
0.6 μg/ml + S9	9.10	1.72	9.50	1.72	9.50	1.75	9.37 ± 0.23***	1.73 ± 0.02***
PC (MMC) (2 μg/ml)	1.16	1.50	11.16	1.45	11.18	1.46	11.17 ± 0.01***	1.47 ± 0.03***
PC(CP.1.4 μg/ml) + S9	12.10	1.46	12.60	1.45	12.50	1.44	12.40 ± 0.26***	1.45 ± 0.01***

SCE: sister chromatid exchanges; NC: negative control; M: Metaphase; CP: cyclophosphamide; MMC: Mitomycin C; TH: thimerosal.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
The MI values in human lymphocyte treated with thimerosal (TH) in cultures with and without S9 *in vitro*

Treatments	Donor A MI (‰)	Donor B MI (‰)	Donor C MI (‰)	Mean ± SD MI (‰)
NC	7.65	7.75	7.75	7.71 ± 0.05
NC + S9	6.00	5.66	6.00	5.88 ± 0.19
Thimerosal 0.2 μg/ml	7.60	7.60	7.80	7.66 ± 0.711
0.2 μg/ml + S9	5.66	5.66	5.33	5.55 ± 0.19*
0.4 μg/ml	5.90	5.70	5.60	5.73 ± 0.15*
0.4 μg/ml + S9	5.00	5.00	4.00	4.66 ± 0.57**
0.6 μg/ml	3.95	4.00	3.90	3.95 ± 0.05**
0.6 μg/ml + S9	4.33	3.66	4.66	4.21 ± 0.50**
PC(MMC) (2 μg/ml)	3.16	3.11	3.32	3.19 ± 0.10***
PC(CP) 1.4 μg/ml + S9	2.33	2.66	3.00	2.66 ± 0.33***

Negative control; PC: Positive control; MMC: Mitomycin C; CP: Cyclophosphamide; MI: Mitotic index.
* $p < 0.05$.
** $p < 0.01$.

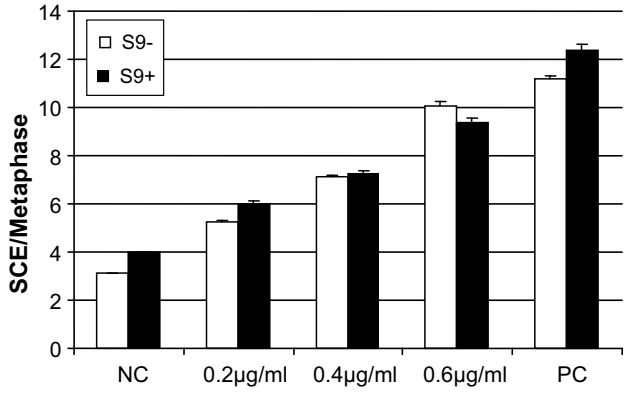

Fig. 2. Frequency of sister chromatid exchanges in relation to thimerosal doses in human peripheral blood cultures. SCE: sister chromatid exchanges; NC: negative control and PC: positive control.

values, significant differences were observed between negative control and cultures treated with TH under conditions with S9 and without S9 fraction ($p < 0.05$ for 0.2 μg/ml $p < 0.001$for 0.4 and 0.6 μg/ml). In all the doses, there is

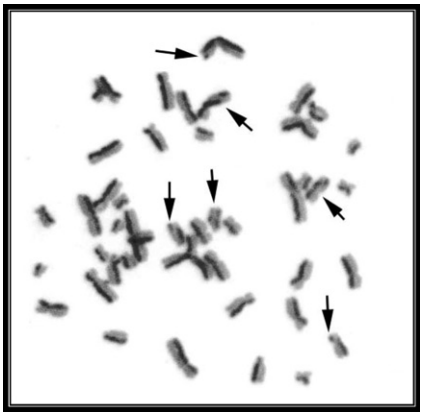

Fig. 3. Arrows show sister chromatid exchanges points.

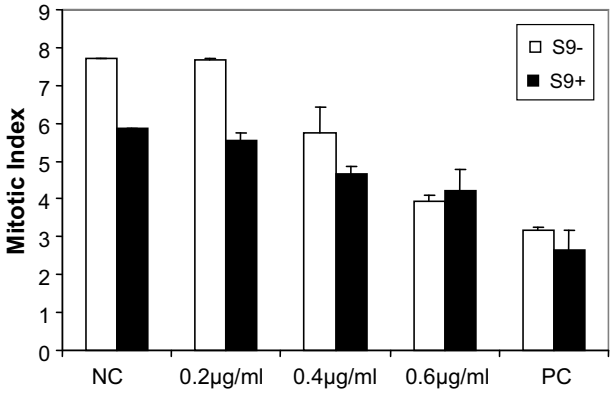

Fig. 4. Frequency of Mitotic index in relation to thimerosal doses in human peripheral blood cultures. NC: negative control; PC: positive control and MI: mitotic index.

no significant difference between condition with S9 and without S9 fraction ($p > 0.05$). These results confirm the possible non-detoxification of the compound mediated by the metabolism of the S9 enzymes, as indicated for SCE study. Fig. 5 demonstrates dose–response characteristics for PRI.

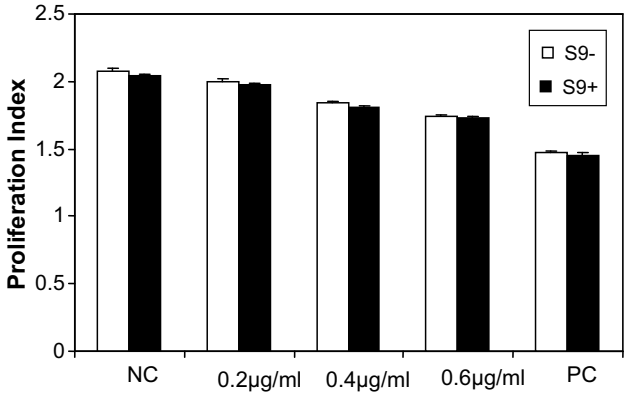

Fig. 5. Frequency of PRI in relation to thimerosal doses in human peripheral blood cultures. NC: negative control; PC: positive control and PRI: proliferation index.

4. Discussion

Genotoxic compounds damage DNA by several different mechanisms (Lindahl and Wood, 1999 and Friedberg et al., 1995). Prooxidant compounds such as Cr(VI) and hydrogen peroxide contribute to the production of oxyradicals. These radicals can induce DNA strand breaks by directly interacting with the sugar molecule, producing sugar damage (Norbury and Hickson, 2001). They can also oxidize bases which can lead to strand breaks and eventually to mutations (Marnett, 2000). DNA ligation, in which the broken strand is edited for integrity, reannealed and ligated (Norbury and Hickson, 2001), is the mechanism for repair of single DNA strand breaks, while oxidized bases can be repaired by direct reversal, when an enzyme restores the DNA damage in a single step (Friedberg et al., 1995), or base excision repair (Lindahl and Wood, 1999 and Friedberg et al., 1995). Adduct formation occurs when a genotoxicant, such as metabolites of certain polycyclic aromatic hydrocarbons, bind directly to the DNA which can distort the DNA helix, preventing transcription and leading to mutations (Friedberg et al., 1995). Lesions such as bulky adducts are repaired by nucleotide excision repair (Sekelsky et al., 2000). Other DNA lesions include protein–DNA crosslinks, DNA–DNA crosslinks and mismatched bases (Friedberg et al., 1995).

Thimerosal (TH) is one of the most widely used preservatives and is found in a variety of biological products, including vaccines, contact lens cleaning solutions and cosmetics. The source of mercury in vaccines is the antimicrobial preservative, Thimerosal, containing 49.9% ethyl mercury by weight. All forms of mercury are well known to be toxic to cells (Costa et al., 2004). Mercury species, including TH, are reported to have effects on the antioxidant status of various cell types including astrocytes and neurons (James et al., 2005; Sanfeliu et al., 2001), lymphocytes (Ueha-Ishibashi et al., 2004), and thymocytes (Macho et al., 1997). Previous mechanistic studies of methylmercury in various cells toxicity have implicated reactive oxygen species (ROS) and depletion of intracellular glutathione as a contributor to mercury-induced cytotoxicity (Sanfeliu et al., 2001). Glutathione provides the major intracellular defense against ROS and oxidative stress-induced cell damage and apoptosis (Meister, 1995). Its depletion was shown to precede the increase in ROS associated with the loss of viability and apoptosis (Macho et al., 1997). Previous studies have demonstrated that thimerosal neurotoxicity is associated with glutathione depletion (James et al., 2005; Ueha-Ishibashi et al., 2004) and this action is likely to be related to (1) changes in cellular redox status modulating channel and receptor activities (Choi and Lipton, 2000; Pessah, 2001) and (2) cell growth and death related to cellular redox state (Hampton and Orrenius, 1998; Mates et al., 2002).

There are no sufficient evidence in relation to TH genotoxicity or mutagenicity and toxicity. This study is the first report on detecting the genotoxic and/or mutagenic effects of TH using SCE assay in human lymphocytes *in vitro*. It is generally acknowledged that the SCE test is an indicator test of genotoxic exposure, whereas the MN assay represents a mutagenicity test for the detection of chromosome mutations. Therefore, the results of the MN assay should be considered of higher significance than the results of an SCE test. Moreover, the biological significance of an increase in SCE in the processes of mutagenesis and carcinogenesis is not clear. SCE is the visible evidence of DNA damage and subsequent homologous recombination, a condition expected to be more compatible with events dependent upon cell survival (e.g. mutagenesis) (Speit and Henderson, 2005). In this study, TH with and without S9 fraction increased the SCE frequencies (Table 1). TH has shown genotoxic activity in different cell lines. Some studies identified the DNA damage or genotoxic effects of TH in different cell cultures and *in vivo* systems (Baskin et al., 2003; Westphal et al., 2003). Baskin et al. (2003) investigated the toxicity of micromolar and nanomolar concentrations of TH (125 nM–250 μM) occurring in the first 24 h of exposure in cultures of human cortical neuronal cells and in human fibroblasts and found that thimerosal in micromolar concentrations rapidly decreased cellular viability. They reported that neuronal cell cultures demonstrated a higher sensitivity to TH compared with fibroblasts. Westphal et al., (2003) studied the genotoxicity of TH on the human lymphocytes using Cytochalacin B Block Micronucleus test and the relationship between genotoxicity and the glutathione-*S*-transferase polymorphism. In this study, blood samples of six healthy donors of different glutathione-*S*-transferase genotypes were included. However, there was no association observed between genotoxicity and the glutathione-*S*-transferase polymorphism investigated. Strong variations in the genotoxic response to thimerosal were seen, but no evidence of the involvement of the GSTM1 genotype was observed. Inter-individual differences in the response were not linked to different GST genotypes. Thimerosal induced a significant increase in micronuclei in primary human lympho-

cytes at non-toxic concentrations between 0.05 and 0.5 μg/ml.

In one study, possible carcinogenic effects of thimerosal were investigated by Mason et al. (1971) on the chronic toxicity in Fischer 344 rats. In accordance with the intended use, thimerosal was applied by subcutaneous injection (0.1, 0.3, and 1.0 mg/kg body weight in 250 μl physiological saline, twice weekly for 12 months). Half of the animals of each experimental group were killed after 12 months and the other animals were kept for an additional 6 months. A non-significant increase of tumors was found in the high-dose thimerosal group. Aberer and Kränke (1995) indicated that thimerosal is a contact-sensitizer. Electrophilicity and the ability to form adduct with proteins and DNA is a common characteristic of some contact-sensitizers and genotoxic carcinogens. Thus, the National Toxicology Program of the USA evaluated 146 chemicals that had been studied for tumorigenicity and mutagenicity. About 20–28% of contact-sensitizers were found to be mutagenic and/or tumorigenic (Albert and Magee, 2000). As indicated by Albert (1997), electrophilicity may cause tumor initiation, tumor progression and mutagenicity via DNA adduction and protein adduction. In addition, protein adduction leads to tumor promotion by cytotoxicity and protein adduction and contact sensitization.

It is well documented that one of the most major cellular response observed both *in vitro* and *in vivo* after the exposure to chemicals, ionizing radiation, and/or other genotoxic agents is the inhibition or the delay of cell-cycle progression. MI and RI are used as indicators of sufficient cell proliferation and cytotoxicity (Anderson et al., 1988; Scott et al., 1991). The interaction between cytotoxicity and tumor promotion was indicated in several studies performed by many researchers (Albert and Magee, 2000). Also cytotoxicity causes the tumor promotion via inflammation and/or humoral immunity. TH exhibits a cytotoxic effect *in vitro* because of its inhibition of mitotic activity. The decrease in the mitotic activity is dose dependent. The frequency of MI decreased with increasing concentrations of TH, (Fig. 4) and statistically significant differences from the control were observed, except at the dose of 0.2 μg/ml ($p > 0.05$) compared with negative control. Similar to our results, high cellular toxicity (decrease in mitotic index) was recently reported in another cell culture models. Makani et al. (2002), investigated the effects on the reactive oxygen species (ROS) formation in the T cells. They reported that thimerosal induced the oxidative stress via ROS and reduced the intracellular level of glutathione and showed that the effect of thimerosal was due to its Hg content. Similar effects have been observed with methylmercury and other inorganic mercury compounds. (Hultberg et al., 1998; Shenker et al., 1998, 1999). The results of the study performed by Silva-Pereira et al. showed that low concentrations of methylmercury chloride and mercury chloride might be cytotoxic in human peripheral blood lymphocytes *in vitro* due to a decrease in the mitotic index. The authors also reported that methylmercury chloride and mercury chloride is genotoxic. They found a significant increase in the relative frequency of chromosome aberrations for all concentrations of the methylmercury chloride when compared with control whether alone or in an evident synergistic combination with mercury chloride. The frequency of polyploidy cells was also significantly increased when compared to control after exposure to all concentrations of methylmercury chloride alone or in combination with mercury chloride. Mercury compounds induce a general collapse of antioxidant mechanisms in the cell by binding to the sulfhydryl groups of glutathione, a radical scavenger. Such a collapse results in cell degeneration and loss of membrane integrity. The binding of this metal to sulfhydryl groups of glutathione blocks its function as a radical scavenger. Thus, free radicals cause DNA damages.

We showed that the concentrations of thimerosal that induced toxic effects in human peripheral blood lymphocytes ranged from 0.2 μg/ml to 0.6 μg/ml. In PRI values, significant differences were observed between negative control and cultures treated with TH ($p < 0.001$). A significant decrease in PRI is an indicator of cytostatic effect. Our results have demonstrated that there are modifications on CPK values expressed as PRI in lymphocyte cultures exposed to TH. Therefore, it may be said that TH has cytostatic effects on human peripheral blood lymphocyte cultures *in vitro* because it causes delay in the cell cycle. As indicated by many researchers, thimerosal affects the proliferation in several cell types decreasing the proliferation rate (Chikahisa and Oyama, 1992; Sarafian, 1993). Ueha-Ishibashi et al., (2005) reported that thimerosal increased intracellular Ca^{2+} concentrations and induced membrane changes in living cells. Studies into the relationship between SCE induction and different expressions of cytotoxicity have shown that SCE enhancement is often accompanied by reduced cell survival and changes in cell-cycle kinetics, as shown by the lower values of MI and PRI (Mourelatos, 1996; Mourelatos et al., 1988).

Inorganic and organic mercurials, including thimerosal, have been shown to increase reactive oxygen species (ROS) and induce a state of "oxidative stress" in numerous cell types; however, it is unknown whether this is a direct effect of the metal. Studies have shown that thimerosal causes release of calcium from intracellular stores followed by an influx of extracellular calcium (Gericke et al., 1993; Elferink, 1999), which can lead to an increase in reactive oxygen and nitrogen species (RNS) production in the mitochondria. The increase in $[Ca^{2+}]i$ is one of the common features in cytotoxicity. Both ROS and RNS have been shown to cause apoptotic cell death (McGowan et al., 1996; Szabo, 1996; Cai and Jones, 1998; Chung et al., 2001). Reactive oxygen species and RNS can directly interact with macromolecules such as proteins and nucleic acids, leading to perturbations of vital cellular functions which may result in cell death.

However, the concentration of mercury in the blood of infants and children receiving vaccines with thimerosal has been reported to be very low, without any toxic effects (Madsen et al., 2003; Bigham and Copes, 2005; Clements and McIntyre, 2006), and the agent is stil recommended as a cheap and stable preservative for vaccines. Countries such as Japan and United States are now tending to reduce the application of thimerosal as much as possible, but it continues to be employed for influenza, tetanus, hepatitis B, poliomyelitis, and measles vaccines in Japan.

In summary, we have shown that thimerosal can cause the mutagenic effect in human blood lymphocytes increasing the SCE frequency and toxic effects decreasing the mitotic index and/or PRI. To our knowledge, this is the first study to show mutagenic and/or genotoxic effect based on SCE assay. Clearly, there is a need for more detailed research on the effects of thimerosal in the body, especially in central nervous system. Further evaluation will be required to determine the relationship between toxic effect, genotoxic effect and apoptotic effect in human peripheral blood lymphocytes and different cell lines *in vitro* and *in vivo*.

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References

Aberer, W., Kränke, B., 1995. Thimerosal is a frequent sensitizer but is not in the standard series. Contact Dermatitis 32, 367–368.

Adler, I.D., Kliesch, U., Van Hummelen, P., Kirsch-Volders, M., 1991. Mouse micronucleus tests with known and suspect spindle poisons: results from two laboratories. Mutagenesis 6, 47–53.

Albert, R.E., 1997. Allergic contact sensitizing chemicals as environmental carcinogens. Environmental Health Perspective 105 (9), 940–948.

Albert, RE., Magee, PS., 2000. The tumorigenicity of mutagenic contact-sensitizing chemicals. Risk Analysis 20, 317–325.

Anderson, D., Jenkinson, P.C., Dewdeney, R.S., Franis, A.J., Godbert, P., Butterworth, K.R., 1988. Chromosome aberrations, mitogen-induced blastogenesis and proliferative rate index in peripheral lymphocytes from 106 control individuals of U.K. population. Mutation Research 204, 407–420.

Aydemir, N., Serap, Ç., Bilaloğlu, R., 2005. In vitro genotoxic effects of the anticancer drug gemcitabine in human lymphocytes. Mutation Research 582, 35–41.

Bal, F., Sahin, F.I., Yirmibes, M., Balcı, A., Menevse, S., 1998. The in vitro effect of bcarotene and mitomycin C on SCE frequency in Down's syndrome lymphocyte cultures. Tohoku Journal Experimental Medicine 184, 295–300.

Ball, LK., Ball, R., Pratt, RD., 2001. An assessment of thimerosal use in childhood vaccines. Pediatrics 107, 1147–1154.

Baskin, D., Ngo, H., Didenko, VD., 2003. Thimerosal induces DNA breaks, Caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts. Toxicological Sciences 74, 361–368.

Ben-Ozer, E., Rosenspire, A., McCabe, M., Worth, R., Kindzelskii, A., Warra, N., Petty, H., 2000. Mercuric chloride damages cellular DNA by a non-apoptotic mechanism. Mutation Research 470, 19–27.

Bigham, M., Copes, R., 2005. Thiomersal in vaccines: balancing the risk of adverse effects with the risk of vaccine-preventable disease. Drug Safe: International Journal Medical Toxicology. Drug Experience 28, 89–101.

Cai, J., Jones, D.P., 1998. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. Journal Biological Chemistry 273 (19), 11401–11404.

Çelik, A., Akbaş, E., 2005. Evaluation of sister chromatid exchange and chromosomal aberration frequencies in peripheral blood lymphocytes of gasoline station attendants. Ecotoxicology and Environmental Safety 60, 106–112.

Chikahisa, L., Oyama, Y., 1992. Tri-n-butyltin increases intracellular Ca^{2+} in mouse thymocytes: a flow-cytometric study using fluorescent dyes for membrane potential and intracellular Ca^{2+}. Pharmacology and Toxicology 71, 190–195.

Choi, Y.B., Lipton, S.A., 2000. Redox modulation of the NMDA receptor. Cellular and Molecular Life Science 57, 1535–1541.

Chung, H.T., Pae, H.O., Choi, B.M., Billiar, T.R., Kim, Y.M., 2001. Nitric oxide as a bioregulator of apoptosis. Biochemical and Biophysical Research Communications 282 (5), 1075–1079.

Clements, C.J., McIntyre, P.B., 2006. When science is not enough—a risk/benefit profile of thimerosal-containing vaccines. Expert Opinion on Drug Safety 5, 17–29.

Costa, LG., Aschner, M., Vitalone, A., Syversen, T., Soldin, OP., 2004. Developmental neuropathology of environmental agents. Annual Review Pharmacology and Toxicology 44, 87–110.

Elferink, J.G., 1999. Thimerosal: a versatile sulfhydryl reagent, calcium mobilizer, and cell function-modulating agent. General Pharmacology 33 (1), 1–6.

Ferrat, L., Romeo, M., Gnassia-Barelli, M., Pergent-Martini, C., 2002. Effects of mercury on antioxidant mechanisms in the marine phanerogam *Posidonia oceanica*. Disease of Aquatic Organisms 50, 157–160.

Friedberg, E.C., Walker, G.C., Siede, W., 1995. DNA Repair and Mutagenesis. ASM Press, Washington, DC.

Gericke, M., Droogmans, G., Nilius, B., 1993. Thimerosal induced changes of intracellular calcium in human endothelial cells. Cell Calcium 14 (3), 201–207.

González Borroto, J.I., Creus, A., Marcos, R., 2002. Genotoxic evaluation of the furylethylene derivative 1-(5-bromofur-2-yl)-2-nitroethene in cultured human lymphocytes. Mutation Research 519, 179–185.

Hampton, MB., Orrenius, S., 1998. Redox regulation of apoptotic cell death in the immune system. Toxicology Letters 102–103, 355–358.

Hoerauf, K.H., Schrögendorfer, K.F., Wiesner, G., Gruber, M., Spacek, A., Kress, H.G., Rudiger, H.W., 1999. Sister chromatid exchange in human lymphocytes exposed to isoflurane and nitrous oxide *in vitro*. British Journal Anaesthesia 82, 268–270.

Holz, O., Scherar, G., Brodtmeier, S., Koops, F., Warnke, K., Krause, T., Austen, A., Angerer, J., Tricker, A.R., Adlkofer, F., et al., 1995. Determination of low level exposure to volatile aromatic hydrocarbons and genotoxic effects in workers at astyrene plant. Occupational and Environmental Medicine 52, 420–428.

Hultberg, B., Anderson, A., Isaksson, A., 1998. Alterations of thiol metabolism in human cell lines induced by low amounts of copper, mercury or cadmium ions. Toxicology 126, 203–212.

Ivett, J.L., Tice, R.R., 1982. Average generation time: a new method of analysis and quantitation of cellular proliferation kinetics. Environmental Mutagenesis 4, 358.

James, S.J., Slikker, W., Melnyk, S., New, E., Pogribna, M., Jernigan, S., 2005. Thimerosal neurotoxicity is associated with glutathione depletion: protection with glutathione precursors. Neurotoxicology 26, 1–8.

Krause, T., Scholz, J., Boettcher, H., Wappler, K.C., Schutle am Esch, J., 2003. Sevoflurane anaesthesia does not induce the formation of sister chromatid exchanges in peripheral blood lymphocytes of children. British Journal Anaesthesia 90, 233–235.

Lindahl, T., Wood, R.D., 1999. Quality control by DNA repair. Science 286, 1897–1905.

López-Nigro, M.M., Palermo, A.M., Mudry, M.D., Carballo, M.A., 2003. Cytogenetic evaluation of two nitroimidazole derivatives. Toxicology in Vitro 17, 35–40.

Macho, A., Hirsch, T., Marzo, I., Marchetti, P., Dallaporta, B., Susin, S.A., Zamzami, N., Kroemer, G., 1997. Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. The Journal of Immunology 158 (10), 4612–4619.

Madsen, K.M., Lauritsen, M.B., Pedersen, C.B., Thorsen, P., Plesner, A.-M., Andersen, P.H., Mortensen, P.B., 2003. Thimerosal and the occurrence of autism: negative ecological evidence from Danish population-based data. Pediatrics 112, 604–606.

Makani, S., Gollapudi, S., Yel, L., Chiplungar, S., Gupta, S., 2002. Biochemical and molecular basis of thimerosal-induced apoptosis in T-cells: major role of mitochondrial pathway. Genes and Immunity 3, 270–278.

Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361–370.

Marques, S., Oliveira, N.G., Chaveca, T., Rueff, J., 2002. Micronuclei and sister chromatid exchanges induced by capsaicin in human lymphocytes. Mutation Research 517, 39–46.

Marrazzini, A., Betti, C., Bernacchi, F., Barrai, I., Barale, R., 1994. Micronucleus test and metaphase analyses in mice exposed to known and suspected spindle poisons. Mutagenesis 9, 505–515.

Mason, M.M., Cate, C.C., Baker, J., 1971. Toxicology and carcinogenesis of various chemicals used in the preparation of vaccines. Clinical Toxicology 4, 185–204.

Mates, J.M., Perez-Gomez, C., Nunez de Castro, I., Asenjo, M., Marquez, J., 2002. Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. International Journal Biochemistry and Cell Biology 31, 697–703.

McGowan, A.J., Ruiz-Ruiz, M.C., Gorman, A.M., Lopez-Rivas, A., Cotter, T.G., 1996. Reactive oxygen intermediate(s) (ROI): common mediator(s) of poly(ADP-ribose) polymerase (PARP) cleavage and apoptosis. FEBS Letters 392 (3), 299–303.

Meister, A., 1995. Glutathione metabolism. Methods Enzymology 251, 3–7.

Miller, J.A., 1970. Carcinogenesis by chemicals: an overview, G.H.A. Clowes Memorial Lecture. Cancer Research 30, 559–576.

Moorhead, P.S., Novell, W.J., Wellman, D.M., Batlips, D., Hungerford, A., 1960. Chromosome preparations of leucocytes cultured from peripheral blood. Experimental Cell Research 20, 613–616.

Mourelatos, D., 1996. Chromosomes study as predictor of chemoresponse of tumours. Cancer Journal 9 (3), 136–141.

Mourelatos, D., Dozi-Vassiliades, J., Kotsis, A., Goutsas, C., 1988. Enhancement of cytogenetic damage and of antineoplastic effect by caffeine in Ehrlich ascites tumour cells treated with cyclophosphamide in vivo. Cancer Research 48, 1129–1131.

Norbury, C.J., Hickson, I.D., 2001. Cellular responses to DNA damage. Annual Review Pharmacology and Toxicology 41, 367–401.

Perry, P., Wolf, S., 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251, 156–158.

Pessah, I.N., 2001. Ryanodine receptor acts as a sensor for redox stress. Pest Management Science 57, 941–945.

Sanfeliu, C., Sebastia, J., Ki, S.U., 2001. Methylmercury neurotoxicity in cultures of human neurons, astrocytes, neuroblastoma cells. Neurotoxicology 22 (3), 317–327.

Sarafian, T.A., 1993. Methyl mercury increases intracellular Ca^{2+} and inositol phosphate levels in cultured cerebellar granule neurons. Journal of Neurochemistry 61, 648–657.

Scott, D., Galloway, S., Marshall, R., Ishidate, M., Brusick, D., Ashby, J., Myhr, B., 1991. Genotoxicity under extreme culture conditions. Mutation Research 257, 147–205.

Sekelsky, J.J., Hollis, K.A., Eimerl, A.I., Burtis, K.C., Hawley, R.S., 2000. Nucleotide excision repair endonuclease genes in Drosophila melanogaster. Mutation Research 459, 219–228.

Shenker, B.J., Guo, T.L., Shapiro, I.M., 1998. Low level methylmercury exposure causes human T-cell to undergoing apoptosis: evidence of mitochondrial dysfunction. Environment Research 77, 149–159.

Shenker, B.J., Guo, T.L., Insug, O., Shapiro, I.M., 1999. Induction of apoptosis in human T-cells by methyl mercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve. Toxicology and Applied Pharmacology 157, 23–35.

Speit, G., Henderson, L., 2005. Review of the in vivo genotoxicity tests performed with styrene. Mutation Research 589, 67–79.

Szabo, C., 1996. DNA strand breakage and activation of poly-ADP ribosyltransferase: a cytotoxic pathway triggered by peroxynitrite. Free Radical Biology and Medicine 21 (6), 855–869.

Tucker, J.D., Preston, R.J., 1996. Chromosome aberrations, micronuclei, aneuploidy,sister chromatid exchange, and cancer risk assessment. Mutation Research 365, 147–159.

Ueha-Ishibashi, T., Tatsuishi, T., Iwase, K., Nakao, H., Umebayashi, C., Nishizaki, Y., Nishimura, Y., Oyama, Y., Hirama, S., Okano, Y., 2004. Property of thimerosal-induced decrease in cellular content of glutathione in rat thymocytes: a flow cytometric study with 5-chloromethylfluorescein diacetate. Toxicology in Vitro 18, 563–569.

Ueha-Ishibashi, T., Oyama, Y., Nakao, H., Umebayashi, C., Hirama, S., Sakai, Y., Ishida, S., Okano, Y., 2005. Flow-cytometric analysis on cytotoxic effect of thimerosal, a preservative in vaccines, on lymphocytes dissociated from rat thymic glands. Toxicology in Vitro 19, 191–198.

Westphal, G.A., Asgari, S., Schulz, T.G., Bünger, J., Müler, M., Hallier, E., 2003. Thimerosal induces micronuclei in the cytochalasin B block micronucleus test with human lymphocytes. Archives Toxicology 77, 50–55.