

# Cytotoxicity and genotoxicity of low doses of mercury chloride and methylmercury chloride on human lymphocytes *in vitro*

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## Abstract

Mercury is a xenobiotic metal that is a highly deleterious environmental pollutant. The biotransformation of mercury chloride ( $\text{HgCl}_2$ ) into methylmercury chloride ( $\text{CH}_3\text{HgCl}$ ) in aquatic environments is well-known and humans are exposed by consumption of contaminated fish, shellfish and algae. The objective of the present study was to determine the changes induced *in vitro* by two mercury compounds ( $\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$ ) in cultured human lymphocytes. Short-term human leukocyte cultures from 10 healthy donors (5 females and 5 males) were set-up by adding drops of whole blood in complete medium. Cultures were separately and simultaneously treated with low doses (0.1 to 1000  $\mu\text{g/l}$ ) of  $\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$  and incubated at 37°C for 48 h. Genotoxicity was assessed by chromosome aberrations and polyploid cells. Mitotic index was used as a measure of cytotoxicity. A significant increase ( $P < 0.05$ ) in the relative frequency of chromosome aberrations was observed for all concentrations of  $\text{CH}_3\text{HgCl}$  when compared to control, whether alone or in an evident synergistic combination with  $\text{HgCl}_2$ . The frequency of polyploid cells was also significantly increased ( $P < 0.05$ ) when compared to control after exposure to all concentrations of  $\text{CH}_3\text{HgCl}$  alone or in combination with  $\text{HgCl}_2$ .  $\text{CH}_3\text{HgCl}$  significantly decreased ( $P < 0.05$ ) the mitotic index at 100 and 1000  $\mu\text{g/l}$  alone, and at 1, 10, 100, and 1000  $\mu\text{g/l}$  when combined with  $\text{HgCl}_2$ , showing a synergistic cytotoxic effect. Our data showed that low concentrations of  $\text{CH}_3\text{HgCl}$  might be cytotoxic/genotoxic. Such effects may indicate early cellular changes with possible biological consequences and should be considered in the preliminary evaluation of the risks of populations exposed *in vivo* to low doses of mercury.

## Key words

- Methylmercury
- Cytotoxicity
- Genotoxicity
- Mitotic index
- Human lymphocytes
- Chromosome aberrations

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## Introduction

Mercury, one of the most widely diffused and hazardous organ-specific environmental contaminants, exists in a wide variety of physical and chemical states, each of which with unique characteristics of target organ specificity (1). In nature, the different forms of mercury include the metallic form, inorganic compounds as well as alkyl, alkoxy and aryl mercury compounds. Once introduced into the environment, mercury compounds can undergo a wide variety of transformations. In sediments, inorganic mercury ( $\text{HgCl}_2$ ) may be converted into methyl ( $\text{CH}_3\text{HgCl}$ ) and dimethyl ( $\text{CH}_3\text{CH}_2\text{HgCl}$ ) forms by methanogenic bacteria. This bio-transformation constitutes a serious environmental risk, given that  $\text{CH}_3\text{HgCl}$  is the most toxic of the mercury compounds and accumulates in the aquatic food chain, eventually reaching human diets (2).  $\text{CH}_3\text{HgCl}$  has been an environmental concern to public health and regulatory agencies for over 50 years because of its neurotoxicity. Its association with nervous system toxicity in adults and infants near Minamata Bay, Japan, in the 1950's initiated environmental health research inquiries that continue to this day (3). The three modern "faces" of mercury are our perceptions of risk from the exposure of billions of people to  $\text{CH}_3\text{HgCl}$  in fish, mercury vapor from amalgam tooth fillings and  $\text{CH}_3\text{CH}_2\text{HgCl}$  in the form of thimerosal added as an antiseptic to widely used vaccines (4).

Mercury genotoxicity has been usually attributed to its ability to react with the sulfhydryl groups of tubulin, impairing spindle function and leading to chromosomal aberrations and polyploidy (5). Another important mechanism of mercury genotoxicity is its ability to produce free radicals that can cause DNA damage (6,7). *In vivo* studies have demonstrated a clastogenic effect of mercury on people exposed to this element in their work environment, through the consumption of contaminated food, or acciden-

tally. Increased numbers of chromosome alterations and micronuclei have been reported in people who consume contaminated fish (8,9) and in miners and workers of explosive factories (10,11). Negative results were also obtained in some cases (12,13), demonstrating that cytogenetic monitoring of peripheral blood lymphocytes in individuals exposed to mercury from different sources may not be completely specific (5).

The effects of  $\text{CH}_3\text{HgCl}$  contamination have been studied in an increasing way since the outbreaks in Japan and Iraq. Many of these studies had its focus on the neurological effects of  $\text{CH}_3\text{HgCl}$  exposure in adult animals and used high doses of this compound (1900 to 30,000 ppb =  $\mu\text{g/l}$ ) to obtain its most severe effects (14). Most of the *in vitro* studies with lymphocytes also used high doses (250 to 6250  $\mu\text{g/l}$ ) of mercury compounds in order to evaluate its clastogenic effects (15-17).

In the present study, we determined the changes induced in human lymphocyte cultures *in vitro* by two mercury compounds, inorganic  $\text{HgCl}_2$  and organic  $\text{CH}_3\text{HgCl}$ . Low doses were used in order to assess the genotoxic and cytotoxic effects of these compounds at concentrations normally found in nature (40 to 500  $\mu\text{g/l}$  in the earth's crust environment) (2). Furthermore, we intended to obtain additional information which could be used in a preliminary evaluation of the risks to populations exposed *in vivo*.

## Material and Methods

### Chemical agents

$\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$  were purchased from Ultra Scientific® (North Kingstown, RI, USA). Distilled water and ethanol (Sigma, St. Louis, MO, USA) were used to dilute  $\text{HgCl}_2$  and  $\text{CH}_3\text{HgCl}$ , respectively. Ten microliters of each agent were added either separately or together to a 5-ml aliquot of a cell suspension in order to obtain final concentrations of 0.1,

1, 10, 100, and 1000  $\mu\text{g/l}$  for each of the agents used for the cytogenetic tests. Cells treated only with vehicles (distilled water or ethanol) were used as controls. The concentration range used in the present study (0.1 to 1000  $\mu\text{g/l}$ ) corresponded to levels observed normally in nature (low doses) (2).

#### ***In vitro* test with human peripheral blood lymphocytes**

Blood samples were obtained from 10 healthy nonsmokers, 5 females and 5 males, aged 18-30 years, with no recent history of exposure to mutagens. The donors gave informed written consent to participate in the study. Cultures were prepared with 1 ml plasma in 5-ml culture medium consisting of 80% RPMI-1640 medium (Gibco, Paisley, UK), 20% fetal calf serum (Cultilab, Campinas, SP, Brazil) with antibiotics (100 IU penicillin/ml and 100  $\mu\text{l}$  streptomycin/ml, Gibco) and 4% phytohemagglutinin (Cultilab). The previously cited concentrations were added to each culture 9 h after the beginning of incubation. For cytogenetic analysis, the lymphocyte cultures were incubated in a water-bath at 37°C for 48 h. Colchicine (0.8 mM; Sigma) was added to the cultures 2 h before harvest to obtain a maximum number of cells at metaphase.

Cells were harvested by centrifugation (300 g), treated for 10 min with 0.075 M KCl (Merck, Darmstadt, Germany), and fixed with 1:3 Carnoy fixative (glacial acetic acid: absolute methanol). Slides were prepared, air-dried and stained for 10 min with 3% Giemsa stain (Merck) diluted in buffer solution, pH 6.8.

The slides were coded and scored in a blind manner using light microscopy. One thousand metaphases per treatment were observed for the analysis of chromosome abnormalities (gaps and breaks). The mitotic index was calculated by counting a total of 3000 cells at each concentration using the formula  $\text{mitotic index} = (\text{number of cells in}$

$\text{division}/\text{total number of cells}) \times 100$ . The polyploidy index was calculated by counting a total of 1000 cells (regardless of their stage in the cell cycle) at each concentration, using the formula  $\text{polyploidy index} = (\text{number of polyploid cells}/\text{total number of cells}) \times 100$ .

Two statistical tests were used for the analysis of chromosome abnormalities: chi-square for the proportion of abnormal cells, and Mann-Whitney U-test for the frequency of gaps and breaks (total number of abnormalities per 100 cells). The Statistica Stat Soft software was used for statistical evaluation. A cell with two or more abnormalities was counted as one for the chi-square test, but as two or more abnormalities for the Mann-Whitney test. The chi-square test was also used to identify the differences in the frequency of polyploidy and mitotic index between treated cultures and controls.

#### **Results and Discussion**

Table 1 shows that the mean mitotic index obtained from the analysis of the 3000 cells/concentration was from 0.12 to 4.0%. The cytotoxic effects of  $\text{CH}_3\text{HgCl}$  were relatively greater, as demonstrated by a significant dose-related decrease in mitotic index following exposure to this compound alone or in combination with  $\text{HgCl}_2$ . A synergistic cytotoxic effect was observed when cells were exposed simultaneously to both compounds. Such effect was also observed in TK6 cells simultaneously exposed to the same agents (18).

Table 2 summarizes the results of analysis of chromosome aberrations in human peripheral lymphocytes in culture following treatment with different concentrations of mercury compounds and their negative controls. The data obtained from 1000 metaphases analyzed per treatment (100 metaphases/individual) showed a significant increase in the frequency of chromatid gaps and chromosomal alterations for all  $\text{CH}_3\text{HgCl}$  concentrations. The same effect was ob-

Table 1. Mean mitotic index (%) of cells treated with different concentrations of mercury chloride and methylmercury chloride.

Concentration (µg/l)	HgCl <sub>2</sub> (A) (%)	CH <sub>3</sub> HgCl (B) (%)	HgCl <sub>2</sub> + CH <sub>3</sub> HgCl (C) (%)	P (A vs B)	P (A vs C)	P (B vs C)
0	4.09 ± 0.1729	4.18 ± 0.2150	4.22 ± 0.1814	NS	NS	NS
0.1	4.01 ± 0.1450	3.78 ± 0.1752	3.43 ± 0.1889	**	**	NS
1	3.96 ± 0.1776	3.32 ± 0.1814	2.66 ± 0.1265*	NS	**	**
10	3.65 ± 0.1179	2.85 ± 0.1581	1.52 ± 0.1989*	**	**	**
100	3.17 ± 0.1338	2.15 ± 0.2014*	0.58 ± 0.3490*	**	**	**
1000	3.00 ± 0.1247	1.63 ± 0.1889*	0.12 ± 0.1751*	**	**	**

Data are reported as mean ± SD. The cells were incubated in RPMI medium, pH 7.0, at 37°C for 48 h. The mitotic index was calculated for 3000 cells per concentration. HgCl<sub>2</sub> = mercury chloride; CH<sub>3</sub>HgCl = methylmercury chloride.

\*P < 0.05 compared to control (chi-square test); \*\*P < 0.05 for data compared between columns (chi-square test). NS = not statistically significant.

Table 2. Relative frequency of cells with gaps, breaks and gaps plus breaks after exposure to mercury chloride and methylmercury chloride.

Agent	Concentration (µg/l)	Gaps (%)		Breaks (%)		Cells with alterations (%)	
		Number	RF (%)	Number	RF (%)	Number	RF (%)
HgCl <sub>2</sub>	0	6	0.6	1	0.1	7	0.7
	0.1	12	1.2*	2	0.2	14	1.4*
	1	7	0.7	0	0.0	7	0.7
	10	9	0.9	0	0.0	9	0.9
	100	6	0.6	0	0.0	6	0.6
	1000	11	1.1*	2	0.2	13	1.3*
CH <sub>3</sub> HgCl	0	5	0.5	2	0.2	7	0.7
	0.1	128	12.8*	7	0.7	135	13.5*
	1	119	11.9*	7	0.7	126	12.6*
	10	70	7.0*	2	0.2	72	7.2*
	100	33	3.3*	3	0.3	36	3.6*
	1000	118	11.8*	4	0.4	122	12.2*
HgCl <sub>2</sub> + CH <sub>3</sub> HgCl	0	5	0.5	2	0.2	7	0.7
	0.1	79	7.9*	7	0.7	86	8.6*
	1	137	13.7*	7	0.7	144	14.4*
	10	200	20.0*	2	0.2	202	20.2*
	100	214	21.4*	3	0.3	217	21.7*
	1000	244	24.4*	4	0.4	248	24.8*

Data are reported for 10 independent experiments. One thousand cells were examined per treatment. The cells were incubated in RPMI medium, pH 7.0, at 37°C for 48 h. HgCl<sub>2</sub> = mercury chloride; CH<sub>3</sub>HgCl = methylmercury chloride; RF = relative frequency.

\*P < 0.05 compared to control. The chi-square test was used for the proportion of cells with alterations and the Mann-Whitney U-test for the relative frequency of gaps and breaks (total number of abnormalities per 100 cells).

served for the simultaneous treatments, together with a synergistic effect.

The frequency of polyploid aberrations (Table 3) was significantly different between cells treated with CH<sub>3</sub>HgCl and cells treated simultaneously with the two compounds when compared to the respective controls. A dose-dependent increase in the frequency of polyploid cells was observed for these treatments and the lowest levels of polyploid aberrations were observed at the different HgCl<sub>2</sub> concentrations.

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, loss of membrane integrity and finally cell necrosis (6). Necrosis can be indicated by a decrease in mitotic index, as shown by the present results. A decrease in mitotic index followed by an increase in the generation of reactive oxygen species was detected in human blood lymphocytes exposed to CH<sub>3</sub>HgCl (15).

Another mechanism that may contribute to cell death induced by mercury compounds is apoptosis. Shenker et al. (19) reported that CH<sub>3</sub>HgCl caused a significant increase in cytochrome *c* in the cytosol of T cells. In contrast, HgCl<sub>2</sub> did not alter the levels of

cytosolic cytochrome *c*, suggesting that the apoptotic pathway triggered by HgCl<sub>2</sub> compounds is independent of cytochrome *c* release. This effect may justify the higher cytotoxic action of CH<sub>3</sub>HgCl, as observed in the present study. Previous results obtained by our group for TK6 cells support the higher cytotoxic effect of CH<sub>3</sub>HgCl compared to HgCl<sub>2</sub> (18). A higher cytotoxic effect of CH<sub>3</sub>HgCl compared to HgCl<sub>2</sub> was also found by other authors (15) after exposure of human lymphocytes to these compounds.

A number of *in vitro* studies on the genotoxic effects of mercury and its compounds based on cytogenetic tests have been published. Their results suggest that organic compounds are generally more active, in terms of genotoxicity, than inorganic compounds (5,15,18,20). The binding of this metal to sulfhydryl groups of glutathione blocks its function as a free radical scavenger (19). Thus, free radicals become available to cause DNA damage (15). These mechanisms can lead to “double-strand breaks” that can be visualized as the chromatid gaps observed in the present study (21) and/or give rise to more evident chromosome alterations such as breaks, rearrangements, and so on (22).

In the present study, a significant dose-

Table 3. Distribution of cells with polyploid aberrations (%) following exposure to different concentrations of mercury chloride and methylmercury chloride.

Concentration (µg/l)	HgCl <sub>2</sub> (A) (%)	CH <sub>3</sub> HgCl (B) (%)	HgCl <sub>2</sub> + CH <sub>3</sub> HgCl (C) (%)	P (A vs B)	P (A vs C)	P (B vs C)
0	0.4 ± 0.0227	0.6 ± 0.6921	0.6 ± 0.3268	NS	NS	NS
0.1	0.3 ± 0.0325	13.0 ± 1.3546*	13.0 ± 1.2364*	**	**	NS
1	0.2 ± 0.0926	22.2 ± 1.2351*	22.5 ± 1.6329*	**	**	NS
10	0.3 ± 0.0086	24.9 ± 3.1993*	25.6 ± 1.3665*	**	**	NS
100	0.5 ± 0.0634	46.6 ± 3.5369*	46.0 ± 2.3625*	**	**	NS
1000	0.5 ± 0.1436	64.3 ± 1.8961*	67.2 ± 5.2438*	**	**	NS

Data are reported as mean ± SD. The cells were incubated in RPMI medium, pH 7.0, at 37°C for 48 h. The polyploid index was calculated by counting a total of 1000 cells (regardless of their stage in the cell cycle). HgCl<sub>2</sub> = mercury chloride; CH<sub>3</sub>HgCl = methylmercury chloride.

\*P < 0.05 compared to control (chi-square test); \*\*P < 0.05 for data compared between columns (chi-square test).

related increase in the number of cells showing chromosome aberrations was observed after treatment with CH<sub>3</sub>HgCl (Table 2). However, such increase was not linearly related to dose. This effect can be explained by the fact that CH<sub>3</sub>HgCl acts similarly to X-rays (23), increasing DNA damage in a dose-dependent manner until a plateau is reached, with a decrease in damage being observed even though the doses continue to increase. This effect reflects two different phenomena: a dose-dependent increase in the proportion of normal cells whose DNA is damaged and a dose-dependent decrease of the probability that such cells can survive higher exposures (24). Similar results were observed on TK6 cells after exposition to CH<sub>3</sub>HgCl (18).

The increased incidence of polyploidy observed in the present study confirms a characteristic effect of mercury compounds, i.e., their action on the mitotic spindle. The strong affinity of mercury for sulfhydryl groups available in the spindle impairs the function of the latter, leading to mistakes in chromosome segregation during cell division and consequently to polyploidy or aneuploidy (5,8,18). These events can be involved in spontaneous abortion, birth defects, cell transformation, and the process of tumor progression (25).

In the present study, we used a range of mercury doses (0.1 to 1000 µg/l, ppb) considered to be low. Much higher concentrations are found in people who live in contami-

nated areas (2,26). Our results showed that exposure of peripheral blood lymphocytes to low doses of CH<sub>3</sub>HgCl is sufficient for the expression of evident genotoxic and cytotoxic effects. However, caution should be taken in using the present results to estimate the risks for populations exposed to low doses of mercury since the appropriate assessment of the genotoxic potential of an agent requires the use of different assays that will permit the evaluation of different genetic events in different cell types (27).

Despite the existence of constraints in the extrapolation of *in vitro* to *in vivo* data in humans, the cytotoxicity of mercury compounds requires special attention in view of the major damage it causes in cell function, resulting in the inability of cells to proliferate. These disturbances frequently appear long before the manifestation of genotoxic effects, or even in the absence of the latter. Thus, the cytotoxic effect may be considered an earlier indication of cellular damage with possible biological consequences and should be taken into account in the preliminary evaluation of the risks to populations exposed *in vivo*, as already suggested by others (8,18).

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