MERCURY CHLORIDE GENOTOXICITY IN RATS FOLLOWING ORAL EXPOSURE, EVALUATED BY COMET ASSAY AND MICRONUCLEUS TEST

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Mercury is a toxic element which is easily absorbed after ingestion or inhalation and deposited mainly in the kidney. The aim of this study was to evaluate the effects of mercury chloride in rats. Female rats, aged 14 weeks, were receiving mercury chloride in oral doses of 0.068, 0.136, and 0.272 mg kg\(^{-1}\) body weight (b.wt.) for five consecutive days. Three days after the last dose, the animals were killed. The liver and the kidney were dissected and mercury measured using vapour generation atomic absorption spectrometry. The results show a significant increase in mercury mass fraction in the kidney after two higher doses of mercury chloride, while liver mercury burden showed a significant increase only after the highest dose. Blood samples were analysed using the comet assay and supravitaly acridine orange stained micronucleus test. Tail length, tail moment and micronucleus frequency were significantly higher in the treated rats than in control rats, regardless of the dose of mercury chloride, while the difference between the treated groups for both comet and micronucleus parameters was not statistically significant.

KEY WORDS: in vivo micronucleus assay, kidney, liver, mercury

Mercury is naturally occurring in the biosphere, but is also released into the environment by human activity, such as mining, combustion of fossil fuels and other industrial release. It is highly toxic to both eucariotic and prokaryotic cells. Its effects depend on the route of exposure and the nature of the mercury compounds (1). The primary target organ of mercury toxicity is the kidney (renal proximal tubule). The main sites of deposition after inhalation of Hg vapour are the kidneys, the liver and the brain (2). Toxic effects of mercury depend on its chemical form. Organic mercury has been shown to be more toxic than inorganic mercury (3-4). However, due to its ability to convert from inorganic to organic form (5) and the other way around (6), both forms may be found in mercury-polluted environments.

Mercury affects antioxidant mechanisms in the cell, resulting in cell degeneration, loss of membrane integrity and finally cellular necrosis (7). Literature data suggest mercury damages DNA. Betti et al., (8) reported DNA fragmentation in human and rat cells assessed by microgel electrophoresis after in vitro and in vivo treatment with methylmercury chloride (MMC) and dimethyl mercury.

Mercury induces DNA single-strand breaks at low concentrations in mammalian cells (9). Grover et al. (10) reported a dose-dependent increase in comet tail length in Wistar rat leucocytes after oral treatment with HgCl\(_2\). Being more harmful than inorganic mercury compounds, organic mercury compounds cause damage at even lower concentrations. Testing mercury compounds for their ability to induce sister chromatid exchanges (SCEs), Lee et al. (1) demonstrated that phenylmercury acetate at concentrations of 1-30 \(\mu\)mol L\(^{-1}\) caused a significant elevation of SCEs in cultured human lymphocytes in a concentration-dependant manner. Although to a lesser extent, positive findings were also obtained for MMC at a...
concentration of 20 µmol L⁻¹, while mercury chloride did not produce positive results when used in the above concentrations. All tree compounds caused a significant increase in endoreduplicated mitoses, which could be due to the inhibition of spindle tubule assembly. Possible spindle toxicity and clastogenic activity of methylmercury was reported by Amorim et al. (11) in their study of cytogenetic damages in a population living in methylmercury contaminated Amazon Basin. Betti et al. (12) also showed that MMC induced chromosome aberrations and aneuploidy in second metaphases, suggesting that MMC produced chromosome segregation errors.

This study was carried out to evaluate the genotoxicity of mercury chloride in rats and to see whether a combination of the comet assay and micronucleus test could serve as indicators of damage caused by mercury chloride.

MATERIALS AND METHODS

Animals and treatment

The animal study was designed in accordance with the national legislation on laboratory animals and was approved by the Ministry of Agriculture and Forestry of the Republic of Croatia. Thirty-two female Wistar rats, aged 14 weeks, of average weight 172 g at the beginning and 183 g at the end of the experiment, were obtained from the Institute’s breeding farm. The animals were receiving standard feed and drinking water ad libitum. The rats were divided in four groups of eight animals each. Three groups were receiving different oral doses of mercury chloride for five consecutive days. The daily doses were 0.068, 0.136 and 0.272 mg HgCl₂ kg⁻¹ b.wt., totalling 0.340, 0.680 and 1.36 mg HgCl₂ kg⁻¹ b.wt., or 0.25, 0.50 and 1.00 mg Hg kg⁻¹ b.wt., respectively. Mercury chloride was dissolved in deionised water and given by gavage. These doses were chosen because the highest oral dose applied in this study did not produce any visible toxic effect in our earlier experiment in which mercury was given orally for five days. The fourth group served as control. Three days after the animals received the last dose of mercury chloride they were killed by exsanguination from the abdominal aorta in ether anaesthesia. Blood samples were processed and analysed using the comet assay and supravital acridine orange stained micronucleus test. The kidney and the liver were dissected for mercury analysis.

Analysis of mercury in organs

After dissection, about 0.5 g of liver and one kidney were weighed, digested in concentrated nitric acid in closed tubes by heating them to 80 °C in an aluminium block (Digestion System DS40, Tecator, Sweden). Total mercury was analysed using cold vapour atomic absorption spectrometry with an LDC mercury monitor (Milton Roy, Riviera Beach, FL, USA) according to the method described earlier (13).

Comet assay

The alkaline comet assay was performed according to Singh et al. (14), with some modifications. Briefly, 100 µL of 0.5 % low melting point agarose (LMA) (Sigma, USA) containing 10 µL of whole blood was dropped to a fully frosted slide (Surgipath, Richmond, IL, USA) precoated with a 300-µL layer of 0.6 % normal melting point agarose (NMA). Slides were allowed to solidify on ice for 10 minutes. The third layer of 100 µL of 0.5 % LMA was then applied and solidified on ice. The slides were then immersed in a freshly prepared lysis solution (2.5 mol L⁻¹ NaCl, 100 mmol L⁻¹ Na₂EDTA, 10 mmol L⁻¹ Tris, with 1 % Triton X-100 and 10 % DMSO added just before use) at least for one hour at +4 °C. After that, the slides were placed in a freshly prepared alkaline buffer (1 mmol L⁻¹ Na₂EDTA, 300 mmol L⁻¹ NaOH, pH 13) and left for 20 minutes to allow DNA to unwind and alkali-labile sites to show. The electrophoresis took 20 minutes at 25 V and adjusting the current to 300 mA. The slides were then neutralised by adding Tris buffer (pH 7.5) dropwise, and washed three times for 5 min, and were stained with 200 µL of ethidium bromide (2 µL mL⁻¹, Sigma, USA). All preparation steps were performed under dimmed light to prevent additional DNA damage.

The slides were analysed using a fluorescence microscope (magnification 250 x) with an excitation filter of 510-560 nm. Images of 100 randomly selected cells (fifty counts on each duplicate slide) were analysed for each sample. DNA migration was analysed by image analysis (Perceptive Instruments, Comet Assay II, Release 1.02, Suffolk), determining the median tail length (TL) and tail moment (TM).

In vivo micronucleus assay - Supravital staining with acridine orange

Acridine orange (AO) coated slides were prepared according to Hayashi et al. (15). Five microlitres of
Significant differences in the kidney were found between groups treated with two higher doses of mercury chloride and the control group, as well as between the treated groups. In the liver, the group treated with 1.36 mg HgCl$_2$ kg$^{-1}$ significantly differed from the control group and the group treated with 0.34 mg HgCl$_2$ kg$^{-1}$.

Table 2 shows the effects of mercury chloride in rat lymphocytes assessed by the comet assay and micronucleus test. Single cell gel electrophoresis under alkaline conditions was used to detect DNA single-strand breaks (Figure 1). To describe the comet parameters, we used tail length and tail moment. The mean lengths of comet tails in treated animals were in the range 18.66±1.10 to 19.24±8.33. All mercury chloride treated groups showed a statistically significant increase in average TL values.

![Microphotograph of a comet in rat lymphocyte showing damaged DNA. The slides were stained with ethidium bromide and analysed using fluorescence microscopy (magnification 250x). DNA migration was analysed by image analysis (Perceptive Instruments, Comet Assay II, Release 1.02, Suffolk).](image-url)
over controls (14.70±0.37). The mean tail moments ranged between 0.65±0.10 and 0.86±0.19 in treated animals. The obtained values differed significantly from the mean control value (0.27±0.04). The difference between the treated groups for both parameters was not statistically significant.

The frequency of micronucleated reticulocytes (Figure 2) increased after the treatment with mercury chloride. The mean values ranged between 3.38±0.46 and 5.37±0.38, and the mean value for controls was 1.63±0.50. The statistical analysis shows a significant difference between control and all treated groups as well as between groups treated with 0.340 and 1.360 mg HgCl₂ kg⁻¹.

**DISCUSSION**

Numerous studies deal with the evaluation of genotoxic effects of mercury and mercury compounds on mammalian cells in culture and experimental animals, as well as in exposed human populations. Results point to an inhibition of DNA synthesis (16), DNA damage (10), inhibition of spindle microtubule assembly (4), reduction in the frequency of mitosis (17), endoreduplication (1), and chromosomal damages (11, 18).

The comet assay showed to be a highly sensitive technique in the evaluation of DNA damage. Little is known at the molecular level about the genotoxic effects following acute exposure of eucariotic cells to low concentrations of mercury. Literature reports a dose-dependent effect of monomethylmercury on the human cells, assessed using the comet assay (19, 20). Because of mercury accumulation in aquatic predators and marine fish and mammals, these organisms are often investigated for mercury-induced genotoxic effects. Other authors (21, 22) showed that methylmercury induced dose-dependent DNA single-strand breaks in dolphin leucocytes. Bombail et al. (23) reported increased MN frequency in red blood cells of butterfish sampled along a pollution gradient in the Firth of Forth in Scotland, while the comet assay did not show any significant DNA breakage. Using the comet assay, Grover et al. (10) established a dose-dependent effect of mercury chloride on rat leucocytes after oral intubation with five doses of compound ranging from 0.0054 to 0.0864 mg/kg b.w.t. Our results of the comet assay show a significant increase in DNA damage in rat lymphocytes after oral administration of mercury chloride. All mercury chloride treated groups showed a statistically significant increase in average TL and TM compared to control. Dose-dependent relationships were not observed. It is possible that the used doses were too high to allow determination of dose-response effects.

Mercury compounds induce the disturbance of the mitotic spindle (24) resulting in aneuploidy and polyploidy, which is due to its affinity to sulphhydryl groups in the spindle apparatus (25). The micronucleus test is widely accepted in the evaluation of clastogenic or aneugenic agents in vivo. Organomercurial compounds showed to increase micronucleus frequency in pregnant mice and their foetuses after intraperitoneal administration of o-hydroxymercuribenzoic acid anhydride, confirming placental transfer of mercury (26, 27). Investigations on aquatic animals, as particularly exposed organisms resulted with controversial findings. Nepomuceno et al. (28) found a significant increase in micronuclei in fish erythrocytes after in vivo exposure to metallic mercury at concentrations of 20 and 200 mg L⁻¹ Hg, but not at lower concentrations. Alsabti (29) described a dose-dependent increase in MN frequency after in vitro mercury and methylmercury treatment of trout hepatic cells. Some other authors did not find any significant increase in MN in fish exposed to mercury (30, 31). The results can not be compared, however, as different organisms or tissues, as well as different mercury forms and concentrations were tested. Human exposure to mercury is proven to result in genotoxic damage. Queiroz et al. (32) reported an increase in MN incidence in workers who were chronically exposed to mercury in concentrations considered biologically safe for the exposed population.
Acridine orange supravital staining method makes it possible to analyse peripheral blood specimens immediately after sampling. The results in this study support our comet assay findings. A significant increase in MN frequency was observed in mercury chloride treated animals compared to control.

Parallel studies between DNA and cytogenetic damages caused by different genotoxic agents revealed a very strong association between the two types of damage. However, there are differences in the sensitivity of methods. Goethem et al., (33) suggest that the comet assay is more sensitive in detecting DNA damaging potential of X-ray radiation. He et al. (34) arrived at a similar conclusion with chemicals used as mutagens. Cebulska-Wasilewska et al. (35) did not find a difference in sensitivity between the comet assay and cytogenetic tests in human lymphocytes exposed to phenylenediamine (o-PDA) and gamma irradiation. Similarly, both methods showed significant effects of mercury chloride in this study. However, in addition to significant difference between control and exposed rats, the micronucleus test showed a dose-dependent effect, which was not observed with the comet assay. Due to the fast DNA repair, some single-strand breaks may escape detection. This may explain why the comet assay did not reveal significant differences between the treated groups.

To conclude, our findings showed that oral exposure to low concentrations of mercury chloride produce a measurable genotoxic effect. A combination of the two used assays proved a good choice for assessing the genotoxicity of mercury chloride.

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**Sažetak**

**GENOTOKSIČNOST ŽIVINA KLORIDA NAKON ORALNE IZLOŽENOSTI ŠTAKORA VREDNOVANA KOMETNIM TESTOM I MIKRONUKLEUSNIM TESTOM**

Živa je toksičan element koji se nakon ulaska u organizam inhalacijom ili ingestijom odlaže najvećim dijelom u bubrezima. U ovom radu praćen je genotoksični učinak živina klorida na životinje štakora. Životinje u dobi od 14 tjedana podijeljene u tri skupine od po osam životinja primale su oralno različite doze živina klorida, 0,068, 0,136 i 0,272 mg kg⁻¹ tjelesne težine. Četvrta skupina od osam životinja služila je kao kontrola. Tri dana nakon zadnje doze živina klorida životinje su žrtvovane. Maseni udjel žive u jetri i bubrezima mjeren je atomskom apsorpcijskom spektrometrijom. Rezultati pokazuju značajan porast masnog udjela žive u bubrezima nakon izlaganja dvjema višim dozama živina klorida, dok je u jetri značajno opterećenje živom opaženo samo kod najviše koncentracije živina klorida. Uzorci krvi životinja analizirani su kometnim testom i mikronukleusnim testom uzoraka supravitalno bojenih akridin oranžom. Primjena ovih dvaju testova pokazala se dobrim izborom pri procjeni genotoksičnosti živina klorida. Naši rezultati pokazuju da izloženost niskim dozama živina klorida može prouzročiti mjerljive genotoksične učinke. Duljina repa, moment repa i učestalost mikronukleusa značajno su povišeni kod tretiranih životinja u odnosu na kontrolu, ali učinak doze nije opažen.

**KLJUČNE RIJEČI:** bubrezi, in vivo mikronukleusni test, jetra, živa

**REQUESTS FOR REPRINTS:**

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