INDUCTION OF MICRONUCLEI BY CADMIUM CHLORIDE IN AO RATS DEPENDS ON AGE AND SEX

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Cadmium (Cd) is one of the most toxic industrial and metal in the environment which may cause severe genotoxic effects. The aim of the work was an evaluation of genotoxic effects of CdCl₂ in genetically pure Albino Oxford (AO) rats, depending on sex, age and dosage. Experimental animals were treated intraperitoneally with three different concentrations of CdCl₂: 0.5, 1, and 2 mg/kg of CdCl₂, while the control animals received equal volume of sterile phosphate buffered saline. The individuals of both sexes were treated at the age of 3, 6 and 12 month. Frequency of micronuclei formation was evaluated in polychromatic erythrocytes (PCEs), 24h hours after the treatment. The results showed that CdCl₂ caused a concentration-dependent increase of micronucleus frequency. The most significant differences were found between ages of 3/12 and 6/12 months at 0.5 and 1.0 mg CdCl₂ concentrations. Namely, 3 month old males had higher frequency of MNi in comparison to 12 month old males, whereas in females it was the opposite. Likewise, 6 months old males exhibited greater sensistivity to CdCl₂ in comparison to 12 month old rats, and in the females it was the opposite. Sex differences were further confirmed as slightly stronger genotoxic effects in 12 months old females treated with 0.5 and 1 mg/kg of CdCl₂. Therefore, the genotoxic effects of cadmium in AO rats depend on concentration, age and sex.

Key words: cadmium, micronuclei, Albino Oxford rats

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INTRODUCTION

Cadmium (Cd) is one of the most toxic metal in the environment which may cause severe genotoxic effects. It is absorbed via the gastrointestinal tract and lungs and accumulated in various organs mainly the kidneys and liver (WERSHANA, 2001). The distribution of Cd between various tissues depends on many factors. Cd has a great affinity to thiol groups and it can be bound to a low molecular weight protein metallothionein (MT) and to high molecular weight proteins (SWIERGOSZ – KOWALEWSKA, 2001). Cd interferes with Ca$^{2+}$ (THÉVENOD, 2009), also binds to O- and N-containing ligands, and can influence the absorption and distribution of this element and replace it in enzymes (SWIERGOSZ – KOWALEWSKA, 2001). Also, it has been shown that Cd can modulate the biological effects of zinc (JIHEN et al., 2011).

Cadmium chloride inhalation induces systemic DNA damage in several organs as a nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testicle (VALVERDE et al., 2000). Cd is clastogen inducing chromosomal aberrations, micronuclei induction, sister chromatid exchange (SCE) and chromosome loss in human and animal cells (JAHANGIR et al., 2005), primarily, single-strand breaks and alkali-labile sites \textit{in vivo} and \textit{in vitro} (KANG et al., 2013). Cd interacts with DNA directly or indirectly (OLIVEIRA et al., 2012) increasing the risk of cancer (JADHAV et al., 2006). The toxic metals, such as Cd, may lower the genetic stability predominantly by two modes of action: the induction of oxidative DNA damage and the interaction with DNA repair processes (HARTWIG, 1995). Cd-induced oxidative stress plays a major role in aberrant gene expression (QU et al., 2005), as well as DNA repair inhibition and apoptosis induction (XIE and SHAIKH, 2006), influencing both damaging and protective signalling pathways, by unknown mechanisms (SEIFRIED et al., 2007).

In the present investigation, we have determined genotoxic effects of Cd on the samples of bone marrow from both sexes of AO rats, using the so-called micronucleus (MN) test. The bone marrow of laboratory rodents is routinely used for evaluation of clastogenic and/or aneugenic effects (ÇELIK et al., 2009; DJELIĆ et al., 2006; BAJIĆ et al., 2008; DJELIĆ et al., 2008). Based upon the intriguing results of our previous study (POPOVIĆ-BUBIJUK et al., 2013) that showed the ability of CdCl$_2$ to induce micronuclei formation in three months old AO rats, regardless of sex, this study broadened the research by including 6 and 12 months old AO rats.

MATERIALS AND METHODS

Chemicals

CdCl$_2$ (Serva Feinbiochemica GmbH, Germany) was dissolved in required amounts of sterile phosphate buffered saline to prepare three experimental concentrations: 0.5, 1, and 2 mg CdCl$_2$ per kg of body weight. Solutions were sterilized by filtration and stored at 4 °C before administration. Cells were maintained in the RPMI-1640 medium (Sigma-Aldrich, USA) containing fetal calf serum (FCS) (ICN Flow, USA) in final concentration of 5% (v/v). FCS was previously inactivated at 56 °C during 30 min. RPMI-1640 medium was also supplemented with HEPES (20 mM; Invitrogen, USA), NaHCO$_3$ (0.85 g/l; Sigma-Aldrich, USA), L-glutamine (2 mM; ICN Flow, USA), and gentamycine (8 µg/ml, ICN Flow, USA). Diethylether (Betahem, Serbia) was used as an anesthetic prior to animal sacrifices by decapitation. Bone marrow smears on clean glass slides were fixed with absolute methanol (Sigma-Aldrich, USA). May-Grünwald and Giemsa stains (Sigma-Aldrich, USA) were used for erythrocyte staining and MNi visualisation.
Experimental animals

AO rats, weighing 150-300 g, were obtained from the Institute for Medical Research of the Military Medical Academy (MMA) in Belgrade, Serbia. The rats were kept at 25 °C and 12 h light: 12 h dark cycle. Animal were fed granulated food (Veterinary Institute Subotica JSC, Serbia) and supplied with water ad libitum.

All experiments were carried out with the consent of the Ethics Committee of the MMA Institute of Medical Research.

Experimental animals were treated intraperitoneally with three different concentrations of cadmium chloride (CdCl₂): 0.5, 1, and 2 mg CdCl₂ per kg of body weight, while the control animals received equal volume of sterile phosphate buffered saline. Individuals of both sexes aged 3, 6 and 12 month were used in these experiments. Frequency of micronuclei formation was evaluated in polychromatic erythrocytes (PCEs), 24h hours after treatment. For bone marrow preparations, femora were isolated, epiphyses cut off and bone marrow cells flushed out using a needle and 5% FCS in RPMI 1640. The cell suspension was centrifuged for 5 min at 1,000 rpm and sedimented cells resuspended. Fine bone marrow cell smears were prepared from the final cell suspension on clear glass slides. After air-drying (2-4 h) at the room temperature and fixing in absolute methanol (2-3 min), slides were stained using May-Grünwald-Giemsa staining method (SAVKOVIĆ, 1990).

The slide analysis was blinded and performed using a Nikon light microscope.

Statistical analysis

The obtained experimental results were analysed by Student's t-test (concentration dependent increase of MNi) and Z-test (comparisons between various age groups and between males and females). The $P \leq 0.05$ value was considered as statistically significant for all tests used.

RESULTS

The effects of Cd on micronuclei induction in polychromatic RBC bone marrow are shown in Table 1 (female and male rats) of different age. The frequency of micronuclei (MNi) is determined at 1000 polychromatic erythrocytes per animal. All results are given with regard to the group of animals which received equal volume of sterile phosphate buffered saline. There wasn’t any statistical significant difference between the group of animals which received equal volume of sterile phosphate buffered saline and group of animals which weren’t exposed to any treatment.

In animals treated with CdCl₂ (0.5, 1 and 2 mg/kg) there was an increase in frequency of micronuclei (MN) in both sexes of AO strain and all age groups, except for 12 month old males, and 3 month old females treated with 0.5 mg/kg of CdCl₂ (Table 1). In most groups of animals an increase in MNi was related to an increase of CdCl₂ concentrations and age. Application of concentrations of 1 and 2 mg/kg CdCl₂ led to a statistically significant increase in frequency of MNi in bone marrow polychromatic erythrocytes (PCE) in all age groups of both sexes ($p < 0.001$).

The lowest concentration applied (0.5 mg Cd / kg body weight) was effective in 6 and 12 months old female animals, 6 months old male animals ($p < 0.05$) and 3 months old male animals ($p < 0.001$). In three months old female group, this concentration did not cause significant increase in numbers of micronuclei (Table 1).
Table 1. The frequency of MN-RBC in polychromatic bone marrow of both sexes of AO rats in different age groups and CdCl$_2$ concentrations. * $p < 0.05$, **$p<0.001$ (Student’s $t$-test; in comparison to negative control).

<table>
<thead>
<tr>
<th>Cd</th>
<th>AO males</th>
<th>AO females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mon.</td>
<td>6 mon.</td>
</tr>
<tr>
<td>Untreated animals</td>
<td>0.57 ± 0.53 n=7</td>
<td>0.86 ± 1.07 n=7</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.57 ± 0.53 n=7</td>
<td>1.00 ± 1.00 n=7</td>
</tr>
<tr>
<td>0.5 mg/kg b.w.</td>
<td>2.86 ± 0.69 b n=7</td>
<td>2.14 ± 1.07 a n=7</td>
</tr>
<tr>
<td>1 mg/kg b.w.</td>
<td>3.86 ± 0.69 b n=7</td>
<td>3.57 ± 1.13 b n=7</td>
</tr>
<tr>
<td>2 mg/kg b.w.</td>
<td>4.43 ± 0.79 b n=7</td>
<td>4.29 ± 0.95 b n=7</td>
</tr>
</tbody>
</table>

In comparison of genotoxic effects of different concentrations of CdCl$_2$ in various age groups of AO rats we observed different relations (Table 2). Comparison of 3 and 6 month old AO rats showed an absence of statisticallly significant differences between these two age groups, both in males and females. The strongest differences in susceptibility to genotoxic effects of CdCl$_2$ were detected between 3 and 12 month old males, at all three concentrations used. However, in females, these differences were detectable at 0.5 and 2 mg/kg of CdCl$_2$, while at the highest concentration (2 mg/kg) there was no statistically significant difference in genotoxic effects measured by in vivo MN test. Interestingly, younger, 3 month old male rats were more susceptible to genotoxic effects of CdCl$_2$ in comparison to 12 month old males. In contrast, in 12 month old females CdCl$_2$ exhibited stronger genotoxic effects than in 3 month old females. Finally,
comparison between 6 and 12 month old rats showed that younger males were also more prone to
genotoxic effects of CdCl$_2$, but only at the lowest concentration used (0.5 mg/kg), while at highest
concentrations we did not observe differences between those two age groups of males. The results
of comparisons between 6 and 12 month old females were similar as comparison of 3 and 12
month old rats – the genotoxic effects of CdCl$_2$ were more expressed in older females, but this
stands only for 0.5 and 1 mg/kg concentrations, whereas at the highest concentration used in this
study (2 mg/kg) there was no statistically significant difference.

Table 3. Sex differences in genotoxic effects of CdCl$_2$ in AO rats of various age. n.s. – non-significant. NB:
For $|Z_o| > u_\alpha/2$ there is a statistical difference ($u_\alpha/2 = 1.96$) in Z-test.

<table>
<thead>
<tr>
<th>AO males/females</th>
<th>3 mon.</th>
<th>6 mon.</th>
<th>12 mon.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.5 mg/kg b.w.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg b.w.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>2 mg/kg b.w.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

In order to compare possible differences in genotoxic effects of CdCl$_2$ between males and
females, we used the Z-test (Table 3). Interestingly, we observed statistically higher frequencies of
MNi in females only in 12 month old rats treated with 0.5 mg/kg and 1 mg/kg, whereas at the
highest concentration (2 mg/kg) there was no difference between males and females. Finally, in 3
and 6 month old rats we did not observe any statistically significant difference between males and
females, at all experimental concentrations used.

DISCUSSION

The present study aimed to investigate the levels of acute genotoxic effects of cadmium
on genetically pure male and female AO (Albino Oxford) strain of rats (Rattus norvegicus), and
evaluate its age and concentration dependence using micronucleus test. Bone marrow was our
choice Cd target, following the findings of ABRAMSSON-ZETTERBERG et al. (1999) who determined
MN in PCE in samples of rat bone marrow, spleen and peripheral blood, and found the frequency
of micronucleated polychromatic erythrocytes higher in bone marrow and spleen compared with
peripheral blood samples.

It has been shown that Cd$^{2+}$ tends to form tight covalent bonds with DNA. The effects
recorded in this study could be explained by an increase in lipid peroxidation within erythrocyte
membranes, as found by GUTTERIDGE (1995) and STOHS et al. (2001) and decreased glutathione
content, superoxide dismutase, glutathione peroxidase and catalase activity that have the ability to
alter antioxidative defence in cells (CARMEN et al., 2002). Cd$^{2+}$ may also indirectly damage DNA
producing reactive oxygen species (ROS) (KARA et al., 2005; JADHAV et al., 2007; ROOPHA and
LATHA, 2013). HARTWIG (1995) reported that metals such as arsenic, cadmium, lead, nickel and
cobalt cause inhibition of DNA repair processes at low, non-cytotoxic concentrations of the
respective metal compounds, and concluded that even though different steps in DNA repair are
affected by diverse metals, one common mechanism may be the competition with essential metal
ions.
In this investigation, rat bone marrow was used for testing the aneugenic and/or clastogenic effects of Cd. We used MN test to evaluate concentration-, age- and sex-dependence. In all three age groups, we found a positive concentration-dependent increase in the number of micronuclei. When it comes to high concentrations of CdCl$_2$ changes are manifested not only in MNi but there is a damage to mature red blood cells that is seen in the red blood cells, or on its membrane (BEYERSMANN et al., 1997). In vitro, low concentrations of Cd stimulate DNA synthesis, cell multiplication, and malignant transformation (BEYERSMAN et al., 1997). Thus, FAHMY and ALY (2000) showed that CdCl$_2$ causes destruction of red blood precursor cells, but these effects were not fully demonstrated on polychromatic erythrocytes CdCl$_2$. When CdCl$_2$ acted only on erythrocytes, then the number of micronuclei in polychromatic erythrocytes was much higher.

In our investigation, MN test showed that there are differences among age groups of rats. Age-related changes in the immune system include a reduction in clonal expansion and a decrease in the function of antigen-specific T and B cells and antigen-presenting cells (SAMBHARA et al., 2001; HSU et al., 2001). As a result, the frequency of MNi in polychromatic red blood cells of bone marrow AO strain differs significantly by age. As for the age groups, the results suggest that the MN frequency variability of the analysed samples is largely due to an age as a complex factor (VIKRAM et al., 2007). Mammals are sensitive to toxic metals from the environment especially in the early stages of development (WERSHANA, 2001). Three months old male rats were shown to be most sensitive to Cd action and the genetic changes that were examined by the MN test. It should be mentioned that during the process of aging tissues may become more sensitive as MT synthesis is insufficient (KUESTER et al., 2002) to bind the metal. Therefore, the protection from harmful effects of Cd is insufficient. In older rats treated with Cd compounds there is a higher level of mortality due to the toxic effects of Cd (SOGAWA et al., 2001, GUPTA et al., 2004). In addition, depletion of activity of the adipokine tumor necrosis factor (TNF) in circulation, leads to reduced ability of an organism to inactivate reactive metabolites (KARMAR et al., 1998). Disruption of homeostatic mechanisms, as well as an increased incidence of degenerative diseases during ageing may also contribute to more profound toxic and/or genotoxic effects of Cd in elderly animals (DE MAO et al., 2005). Interestingly, in females the situation was opposite — CdCl$_2$ had the strongest effects in 12 month old rats. Moreover, 12 month old females treated with 0.5 or 1 mg/kg of CdCl$_2$ exhibited higher frequency of MNi than males of the same age. We assume that this discrepancy between males and females resulted from differences in sex hormones in their bodies, and this assumption is corroborated by findings of SHIMADA et al. (2012).

Apart from Cd, other heavy metals, ionizing radiation, cyclophosphamide and vincristine can induce DNA damage and appearance of small nuclei (micronuclei) in polychromatic erythrocytes in rat bone marrow, especially in a synergistic action (ABRAMSSON-ZETTERBERG et al., 1999, JADHAV et al., 2006, BROZOVIĆ, 2007, LEWINSKA et al., 2007, TRIPATHI and JENA, 2008, TAPISSO et al., 2009). The use of certain antioxidants such as selenium, vitamin C and E, or royal jelly may help decrease the toxicity of heavy metal ions and contribute to prevention of mutagenesis and carcinogenesis (HURNA and HURNA, 2000, CAVUSOGLU et al., 2009).

The results presented in this paper bring further information about adverse effect of Cd which might get access to the organism and induce genotoxic damage. AO rats showed gross susceptibility to cytogenetic damage by CdCl$_2$. The effects of cadmium on in vivo induction of micronuclei in the bone marrow cells and the cell damage depend on concentration of Cd, age and sex of rats.
ACKNOWLEDGMENTS

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INDUKCIJA MIKRONUKLEUSASA KADMIJUM HLORIDOMU AO PACOVIMA ZAVISNO OD POLA I STAROSTI

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Izvod
Kadmijum (Cd) je jedan od najtoksičnijih metala u spošašnoj sredini koji može da prouzrokuje ozbiljne genotoksične efekte. Cilj ovog rada je evaluacija genotoksičnog efekta CdCl₂ u genetički čistim Albino Oksford (AO) pacovima, zavisno od pola, doba i doza. Eksperimentalne životinje su tretirane intraperitenalnom tri različite koncentracije CdCl₂: 0.5, 1, 2 mg/kg CdCl₂, dok su kontrolne životinje dobile istu zapreminu sterilnog fosfatnog bufera. Individue oba pola su tretirane u uzrastu od 3, 6 i 12. Učestalost formiranja mikronukleusa je ispitana u polihromatnemeritrocitima (PCEs), 24h posle tretmana. Rezultati pokazuju da CdCl₂ uzrokuje koncentracija zavisno povećanje frekencije mikronukleusa. Najznačajnije razlike su nađene između 3/12 i 6/12 meseci sa 0.5 i 1.0 mg CdCl₂. 3 meseca stari mužjaci su imali veću frekenciju MNi u poređenju sa 12 meseci stariim mužjacima, dok je kod ženki bilo suprotno. Slično 6 meseci stari mužjaci su pokazali veću osetljivost CdCl₂ u poređenju sa 12 meseci stariim pacovima i u ženkama je bilo suprotno. Polne razlike su dalje potvrđene kao blago jači genotoksičan efekat u 12 meseci stariim ženkama tretiranih sa 0.5 i 1 mg/kg CdCl₂. Zato, genotoksičan efekat kadmiijuma u AO pacovimazavisni od koncentracije, doba i pola.

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