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

Slavica POPOVIĆ BUBUJUK[1,2], Adrian MUSTER[3]; Ninoslav DJELIĆ[4]; Dragan KATARANOVSKI[5], Marko ANDJELKOVIĆ[6]

[1]Elementary school "Veselin Masleša", Belgrade, Serbia
[2]Faculty of Biofarming, Megatrend University, Bačka Topola, Serbia
[3]Faculty of mechanical engineering, University of Belgrade, Belgrade, Serbia
[4]Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia
[5]Faculty of Biology, University of Belgrade, Belgrade, Serbia
[6] Serbian Academy of Science and Arts, Belgrade, Serbia

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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 $CdCl_2$ in genetically pure Albino Oxford (AO) rats, depending on sex, age and dosage. Experimental animals were treated intraperitoneally with three different concentrations of $CdCl_2$: 0.5, 1, and 2 mg/kg of $CdCl_2$, 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 $CdCl_2$ 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 $CdCl_2$ 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 exbibited greater sensistivity to $CdCl_2$ 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 $CdCl_2$. Therefore, the genotoxic effects of cadmium in AO rats depend on concentration, age and sex.

Key words: cadmium, micronuclei, Albino Oxford rats

Corresponding author: Ninoslav Djelić,Department of Biology,Faculty of Veterinary Medicine University of Belgrade,Oslobodjenja Blvd. 18,11000 Belgrade, Serbia,Phone: +381 11 265 88 94, Fax: +381 11 268 59 36,E-mail: ndjelic@vet.bg.ac.rs

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-containg 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 *in vivo* and *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-BUBUJUK *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 ºC 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; Ivitrogen, 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 staning 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 ºC 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 ($CdCl_2$): 0.5, 1, and 2 mg $CdCl_2$ 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 $CdCl_2$ (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 $CdCl_2$ (Table 1). In most groups of animals an increase in MNi was related to an increase of $CdCl_2$ concentrations and age. Application of concentrations of 1 and 2 mg/kg $CdCl_2$ led to a statistically significant increase in frequency of MNi in bone marrow polychomatic erytrocytes (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. [a] * p <0.05, [b]***p<0.001 (Student□s t-test; in comparison to negative control).*

Cd	AO males			AO females		
	3 mon.	6 mon.	12 mon.	3 mon.	6 mon.	12 mon.
Untreated animals	0.57 ± 0.53 n=7	0.86 ± 1.07 n=7	0.71± 0.76 n=7	0.14 ± 0.38 n=7	0.86± 0.69 n=7	0.57± 0.79 n=7
Negative control (solvent)	0.57 ± 0.53 n=7	1.00 ± 1.00 n=7	0.86 ± 0.,90 n=7	1.86 ± 0.38 n=7	1.14 ± 0.69 n=7	1.6 ± 0.89 n=5
0,5 mg/kg b.w.	2.86 ± 0.69 [b] n=7	2.14 ± 1.07 [a] n=7	1.00 ± 1.00 n=7	2.43 ± 0.53 n=7	2.29 ± 0.76[a] n=7	3.25 ± 0.5 [a] n=5
1 mg/kg b.w.	3.86 ±0. 69 [b] n=7	3.57 ± 1.13 [b] n=7	2.86 ± 0.69 [a] n=7	3.43 ± 0.79 [b] n=7	3.14 ± 0.69 [b] n=7	4,5 ± 1 [b] n=5
2 mg/kg b.w.	4.43 ± 0.79 [b] n=7	4.29 ± 0.95 [b] n=7	3.57 ± 0.53 [b] n=7	4.5 ± 1.05 [b] n=7	3.86 ± 0.9 [b] n=7	4,5 ± 1.04 [b] n=5

Table 2. The frequency of MN-RBC in polychromatic bone marrow of male AO rats in different age groups and $CdCl_2$ concentrations. NB: For $|Z_o| > u\alpha/2$ there is a statistical difference ($u\alpha/2 = 1.96$) in Z-test.

	AO males			AO females		
Cd	3/6 mon.	3/12 mon.	6/12 mon.	3/6 mon.	3/12 mon.	6/12 mon.
0,5 mg/kg b.w.	n.s.	4,04 > 1,96	2,07 > 1,96	n.s.	2,73 > 1,96	2,67 > 1,96
1 mg/kg b.w.	n.s.	2,7 > 1,96	n.s.	n.s.	1,98 > 1,96	2,62 > 1,96
2 mg/kg b.w.	n.s.	2,38 > 1,96	n.s.	n.s.	n.s.	n.s.

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 abscence of statistically 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 diffferences 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 $CdCl_2$ exhbited 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.

	AO males/females		
Cd	3 mon.	6 mon.	12 mon.
0,5 mg/kg b.w.	n.s.	n.s.	5,11 > 1,96
1 mg/kg b.w.	n.s.	n.s.	3,15 > 1,96
2 mg/kg b.w.	n.s.	n.s.	n.s.

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 glutathion 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 concentracions 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 (KARMAKAR *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 MAIO *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 erytrocytes 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.

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INDUKCIJA MIKRONUKLEUSASA KADMIJUM HLORIDOMU AO PACOVIMA ZAVISNO OD POLA I STAROSTI

SLAVICA POPOVIĆ BUBUJUK[1,2], [3]ADRIAN MUSTER; [4]NINOSLAV DJELIĆ; [5]DRAGAN KATARANOVSKI, [6]MARKO ANDJELKOVIĆ

[1]Osnovna škola"Veselin Masleša", Beograd, Srbija
[2]Fakultet za biofarming Megatrend Univerzitety, Bačka Topola, Srbija
[3]Mašinski fakultet, Univerzitet u Beogradu, Beograd, Srbija
[4]Fakultet Veterinarske medicine, Univerzitet u Beogradu, Beograd, Srbija
[5]Biološki fakultet, Univeruitet u Beogradu, Beograd, Srbija
[6] SANU, Beograd, Srbija

Izvod

Kadmijum (Cd) je jedan od najtoksičnijih metala u spošašnjoj sredini koji može da prouzrokuje ozbiljne genotoksične efekte. Cilj ovog rada je evaulacija genotoksičnog efekta $CdCl_2$ u genetički čistim Albino Oksford (AO) pacovima, zavisno od pola, doba I doze. Eksperimentalne životinje su tretirane intraperitenalnosa tri različite koncetracije $CdCl_2$: 0.5, 1, I 2 mg/kg $CdCl_2$, dok su kontrolne životinje dobijale istu zapreminu sterilnog fosfatnog bufera. Individue oba pola su tretirane u uzrastu od 3, 6 i 12. Učestalost formiranja mikronukleusa je ispitana u polihromatnimeritrocitima (PCEs), 24h posle tretmana. rzultati pokaziju da $CdCl_2$ uzrokuje koncetracija zavisno povećanje frekfencije mikronukleusa. Najznačajnije razlike su nađene između 3/12 i 6/12 meseci sa 0.5 i 1.0 mg $CdCl_2$. 3 meseca stari mužjaci su imali veću frekfenciju MNi u poređenju sa 12 meseci starim mužjacima, dok je kod ženki bilo suprotno. Slićno 6 meseci stari mužjaci su pokazali veću osetljivost $CdCl_2$ u poređenju sa 12 meseci starim pacovima i u ženkama je bilo suprotno. Polne razlike su dalje potvrđene kao blago jači genotoksičan efekat u 12 meseci starim ženkama tretiranih sa 0.5 i 1 mg/kg $CdCl_2$. Zato, genotoksičan efekat kadmijuma u AO pacovimazavisi od koncetracije, doba i pola.

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