THE EFFECT OF DIFFERENT ACUTE CONCENTRATIONS OF CADMIUM CHLORIDE ON THE FREQUENCY OF MICRONUCLEI IN AO RATS

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Cadmium (Cd) is highly toxic heavy metal which may cause severe biological effects *in vivo* and *in vitro*. In this study, an evaluation of the acute Cd ability to trigger micronuclei (MNi) formation was carried out on 3-month-old male and female Albino Oxford (AO) rats using micronucleus (MN) test. 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. Control animals received equal volume of sterile phosphate buffered saline. The results showed that 2 mg $CdCl_2$ per kg b.w. concentration caused a highly statistically significant ($P < 0.001$) increase in MNi formation in the bone marrow polychromatic erythrocytes (PCEs), exerting a clear-cut concentration-dependent effect. Lower concentrations of $CdCl_2$ used (0.5 and 1 mg/kg b.w.) also caused MNi formation, but with lower statistical significance. Sex differences in MNi production in bone marrow PCEs after acute exposure to different experimental concentrations of $CdCl_2$ were not observed in our study. Our results indicate the ability of $CdCl_2$ to exerts genotoxic effects in bone marrow of AO rats, and complement previous data on the genotoxicity of this important environmental contaminant, burdening the body from different sources – major being industrial exposure, food and cigarette smoking.

Key words: Albino Oxford rats, cadmium, genotoxicity, micronucleus test

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INTRODUCTION

Cd is one of the most potent hazardous heavy metals in our environment (HU *et al*., 2001), which exhibits toxic (GODT *et al*., 2006), teratogenic (WERSHANA, 2001), mutagenic (BARTOSIEWICZ *et al*., 2001), and carcinogenic effects (ZENG *et al*., 2003).

Cd exposure was reported to produce various direct and indirect genotoxic effects on cells, such as cell proliferation (HUANG *et al*., 2008), chromosomal aberrations (GÜERCI *et al*., 2000), DNA strand breaks (HALDSRUD and KRØKJE, 2009), aberrant DNA methylation (BENBRAHIM-TALLAA *et al*., 2007), and oxidative DNA damage (LIN *et al*., 2007). Cd exposure inhibits DNA synthesis and cell division at concentrations above 1 μM (MISRA *et al*., 2003).

Increased formation of the reactive oxygen species (ROS) in presence of Cd – which can directly generate DNA damage and/or cause inhibition of DNA repair system – is generally recognized mechanism of Cd mutagenicity (FILIPIČ *et al*., 2006). Cd-induced decrease of cellular antioxidants is also considered an important mechanism of Cd contribution to oxidative stress, because it is not redox-active metal and cannot itself direct Fenton reaction (BERTIN and AVERBECK, 2006).

The genotoxicity of Cd *in vivo* and *in vitro* is well documented (SEOANE and DULOUT, 2001; PALUS *et al*., 2003). Cd also shows co-genotoxic effects when combined with other mutagenic agents like UV and gamma radiation, or in presence of alkylating chemicals like methyl methanesulfonate and *N*-methyl-*N*-nitrosourea (FATUR *et al*., 2003).

The bone marrow tissue of laboratory rodents (predominantly mice, *Mus musculus* L.) is routinely used in *in vivo* MN test (ÇELIK *et al*., 2009). Formation of MNi in bone marrow PCEs, which contain lagging chromosome fragments and/or whole chromosomes, is considered a powerful biomarker for clastogenic and/or aneugenic DNA damage (NORPPA and FALCK, 2003; DJELIĆ *et al*., 2006; BAJIĆ *et al*., 2008; DJELIĆ *et al*., 2008). Since the frequency of spontaneously micronucleated cells is generally low (HEDDLE, 1973; SCHMID, 1975; SAVKOVIĆ, 1990), any increase in number of MNi in PCEs represents a relevant evidence of genotoxicity of substance tested (ABRAHIM *et al*., 2011).

It is well shown, that constitutional factors like age, strain, and other genetically determined factors, play important role in Cd induced biological effects (SHIMADA *et al*., 2002; KATARANOVSKI *et al*., 2009).

Taking in consideration that data implying host factors in study of Cd-induced genotoxicity on bone marrow tissue of AO rats are scarce, this study aimed to evaluate an acute Cd effect on groups of 3-month-old male and female AO rats using MN test. Even though, the male rodents alone are sufficient for use in routine bone marrow MN tests (ITOH *et al*., 2012), this study also evaluated potential sex differences associated to CdCl$_2$ exposure in 3-month-old male and female 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.

Ether (Betahem, Serbia) was used as anesthetic prior to animal sacrifices by decapitation.

Bone marrow samples were collected from femurs by flushing the bone marrow plugs with fetal calf serum (FCS) (ICN Flow, USA).

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 visualization.

Experimental animals

Experiments were conducted on 3-month-old male and female AO rats (150-300 g), obtained from the Institute for Medical Research of the Military Medical Academy (MMA) in Belgrade, Serbia. The rats were kept at 25 ºC (12 h light/12 h dark cycle), 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.

Before the administration of $CdCl_2$ animals were set in 8 groups of 7 individuals and acclimated to the laboratory conditions for 5 days. The arrangement of the cages substantially minimized the occurrence of effects due to the cage placement.

Three different concentrations of $CdCl_2$ (0.5, 1, and 2 mg $CdCl_2$ per kg b.w.) were administered in 0.5 ml aliquots to 6 groups of experimental animals, both male and female AO rats. Two groups of AO rats – one for each sex – received 0.5 ml sterile phosphate buffered saline (control animals).

In vivo micronucleus test

The mammalian *in vivo* MN test is used for the detection of damage induced by the test substance to the chromosomes or mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents (OECD, 2012).

When a bone marrow erythroblast develops into PCEs, the main nucleus is extruded and MNi formed may remain in the otherwise anucleated cytoplasm. It is known that PCEs or immature erythrocytes contain RNA. Therefore, they can be distinguished from mature – normochromatic erythrocytes, which are devoided of RNA (OECD, 2012).

The animals were anesthetized with ether and sacrificed 24 hours after the solutions were administered. For bone marrow preparations, femora were isolated, epiphyses cut off and bone marrow plugs flushed out using a needle and small amount of FCS. The cell suspensions were centrifuged for 5 min at 1,000 rpm and sedimented cells were resuspended. Fine bone marrow cell smears were prepared from the final cell suspensions 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).

One thousand of PCEs per animal were counted for the frequency of micronucleated cells (SCHMID, 1975; SAVKOVIĆ, 1990). The slide analysis was blinded and performed using the Nikon Eclipse Ci light microscope (Nikon Instruments, Japan).

Statistical analysis

The results obtained in each experimental group of animals were compared with results obtained in control groups and statistically analyzed using Student's *t*-test and *Z*-test.

RESULTS

The frequencies of MNi in bone marrow PCEs in 3-month-old male and female AO rats, treated with different concentrations of the $CdCl_2$ (0.5, 1, and 2 mg $CdCl_2$ per kg b.w.) are shown in Table 1.

Table 1. The frequencies of MNi in bone marrow PCEs in 3-month-old male and female AO rats treated with different $CdCl_2$ concentrations

$CdCl_2$ (mg/kg b.w.)	Male AO rats		Female AO rats	
	Number of animals	MNi frequency (mean±SD)	Number of animals	MNi frequency (mean±SD)
0 (Negative control*)	7	0.57 ± 0.53	7	1.86 ± 0.38
0.5	7	2.86 ± 0.69**	7	2.43 ± 0.53**
1	7	3.86 ± 0.69**	7	3.43 ± 0.79**
2	7	4.43 ± 0.79***	7	4.50 ± 1.05***

*Control animals received an equal volume of sterile phosphate buffer saline;
**P<0.05; ***P<0.001

Concentration-dependent increase in frequencies of MNi in bone marrow PCEs were detected in all experimental groups, ranging from 2.86±0.69 (0.5 mg/kg b.w.) to 4.43±0.79 (2 mg/kg b.w.) – for male AO rats, and from 2.43±0.53 (0.5 mg/kg b.w.) to 4.5±1.05 (2 mg/kg b.w.) – for female AO rats.

When treated with 2 mg $CdCl_2$ per kg of body weight, experimental animals showed statistically highly significant increase in MNi frequencies (P < 0.001). Lower concentrations of $CdCl_2$ used in this experiment (0.5 and 1 mg/kg b.w.) also exerted genotoxic effects in experimental groups, however, with lower statistical significance (P < 0.05).

In addition, when applying Z-test and considering both – male and female individuals of 3-month-old AO rats as a group – statistically significant difference in MNi production in bone marrow PCEs between experimental groups were observed ($|Z_o| > 1.96$), suggesting that frequencies of MNi production were increased in proportion to the elevation of the concentration of $CdCl_2$ administered (Table 2).

Table 2. Comparison of the frequencies of MNi in bone marrow PCEs when male and famale AO rats are considered as a group

CdCl$_2$ (mg/kg b.w.)	AO rats males + females
0.5	4.93 > 1.96***
1	7.36 > 1.96***
2	8.33 > 1.96***

NB: For $|Z_0| > u\alpha/2$ there is a statistical difference ($u\alpha/2 = 1.96$); ***$P<0.001$

The frequencies of MNi in bone marrow PCEs in 3-month-old male and female AO rats, treated with same experimental concentration of CdCl$_2$, were also compared using Z-test to provide any evidence for potential sex differences in MNi production evoked by an acute CdCl$_2$ treatment. Statistically significant differences were not found in MNi frequencies between male and female AO rats ($1.02 < 1.96$ – for CdCl$_2$ concentration of 0.5 mg/kg b.w., $0.41 < 1.96$ – for CdCl$_2$ concentration of 1 mg/kg b.w., and $0.14 < 1.96$ – for CdCl$_2$ concentration of 2 mg/kg b.w.). Therefore, no sex differences in production of MNi in bone marrow PCEs of 3-month-old AO rats, after acute exposure to different experimental concentrations of CdCl$_2$, were observed in our study.

DISCUSSION

Mammals are sensitive to Cd emanating from the environment especially during the early developmental stages (PILLET *et al*., 2005). Our results also revealed the ability of CdCl$_2$ to induce MNi formation in bone marrow PCEs of 3-month-old male and female AO rats in concentration-dependent fashion.

Studying male Sprague-Dawley rats, HUNDER (2001) found that age affects the distribution of Cd in the body. In adult animals the accumulation is higher in liver, while in the animals in growing phase the peak accumulation values were found in bones, causing subsequential changes in the entire skeletal system. Cd directly affects bones through the changes in bone formation and resorption, and indirectly – via mechanisms involved in Ca metabolism (BRZÓSKA and MONIUSZKO-JAKANIUK, 2005).

Cd can also decrease the activity of TNF- alfa and other inflammatory cytokines in the circulation in terms of a generalized inflammatory response (YAMANO *et al*., 2000). Reduced activity of these molecules can reduce the ability of the organism to inactivate reactive metabolites (ESPINOZA *et al*., 2012), especially through modulating expression of the metallothionein genes (KIMURA and ITOH, 2008).

Effects of 24 h treatment by low Cd concentrations include: increased lipid peroxidation and content of cytosolic free Ca, decreased content of glutatione, superoxide dismutase, glutathione peroxidase and catalase, and thus may alter the antioxidant system of the cells (DEL CARMEN *et al*., 2002). Moreover, an oxidative stress induces DNA damage and interferes with DNA repair processes, inhibiting the repair of DNA lesions affecting DNA replication and cell differentiation (JADHAV *et al*., 2006).

HENGSTLER *et al*. (2003) reported that Cd can cause inhibition of DNA repair, generation of ROS, depletion of glutathione and, possibly, the suppression of apoptosis. The promotion of genetic damage to the cells, together with the suppression of apoptosis, may also induce diverse negative effects in cells and possibly facilitate tumor development (GRASSESCHI *et al*., 2003).

The effect of Cd on cell proliferation has been reported to be concentration dependent, whereby lower concentrations (1 μM) showed stimulating and higher concentrations suppressing effects on the proliferation process (HENGSTLER *et al*., 2003). At noncytotoxic concentrations, Cd interferes with DNA repair processes and detoxifying enzymes, and enhances the genotoxicity as direct-acting mutagen (BEYERSMANN *et al*., 1997). Elevated cytotoxic concentration of Cd inhibits the biosyntheses of DNA, RNA and proteins, and induces lipid peroxidation, DNA strand breaks and chromosome aberrations (BEYERSMANN *et al*., 1997).

Higher $CdCl_2$ concentrations (3 mg/kg b.w.) also cause rapid changes in MNi production in diverse biological systems and damage of mature red blood cells. FAHMY and ALY (2000) showed that $CdCl_2$ can be responsible for red blood precursor cells destruction.

Once accumulated in the cells, Cd inhibits the influx of Ca^{2+} and can also cause a rapid elevation in cytosolic Ca^{2+}, what can be crucial for an induction of Cd-mediated apoptosis (LI *et al*., 2000; WANG *et al*., 2008).

Our results clearly indicate that $CdCl_2$ can exhibit genotoxic effects on bone marrow of 3-month-old male and female AO rats. It is already shown that bone marrow of 3-month-old male and female AO rats proved to be a good biological indicator of the consequential processes (ÇELIK *et al*., 2009). Furthermore, our results are consistent with results obtained in a variety of experiments with different rat strains challenged with Cd, e.g. F344/DuCrj (HAMADA *et al*., 2003), Dark August (POPOVIĆ BUBUJUK, 2009), and Wistar (ÇELIK *et al*., 2009) rats.

Many studies indicate that xenobiotics show different behavior in males and females in many species (e.g., ANGELE *et al*., 2000; BUTTER, 2006).

SHIMADA *et al*. (2012) showed that female sex hormones such as progesterone and β-estradiol are involved in the sexual dimorphism of Cd toxicity in rats. In addition, BRZÓSKA and MONIUSZKO-JAKONIUK (2005) found evidence that young male Wistar rats are less vulnerable to the effects of Cd compared to female Wistar rats.

Sexual dimorphism regarding the sensitivity to Cd exposure has been regularly explained by differential disposition of Cd among different tissues and organs, and/or MT content (VALVERDE *et al*., 2000). Moreover, hormone-dependent tissue distribution of Cd is considered relevant to differences in Cd toxicity among the sexes (BAKER *et al*., 2003; KATARANOVSKI *et al*., 2009).

Even though the sex-related differences in effects regarding the exposure to Cd were proposed by many scientists and explained as a result of protective endocrine effects – which is thought to be mediated through the modulation of MT synthesis (HAMADA *et al*., 2003) – sex dimorphism in production of MNi in bone marrow PCEs after an acute exposure of 3-month-old

male and female AO rats to different experimental concentrations of $CdCl_2$ was not observed in our study. Therefore, more research must be undertaken to elucidate the phenomenon.

CONCLUSION

The experimental results obtained in this study bring further information about adverse effects of Cd, including its potential to cause significant MNi formation in bone marrow PCEs of 3-month-old male and female AO rats. The increase in MNi frequency shows that Cd causes genotoxic effects in male and female AO rats – when administered in these experimental concentrations – and identifies Cd as a mammalian clastogenic and/or aneugenic agent.

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EFEKAT RAZLIČITIH AKUTNIH KONCENTRACIJA KADMIJUM-HLORIDA NA UČESTALOST MIKRONUKLEUSA KOD PACOVA AO SOJA

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Izvod

Kadmijum (Cd) je veoma toksičan teški metal koji može da prouzrokuje ozbiljne biološke efekte *in vivo* i *in vitro*. U ovom istraživanju, procena potencijala Cd da izazove produkciju mikronukleusa (MNA) u polihromatskim eritrocitima (PHE) košatne srži, sprovedena je na tri meseca starim mužjacima i ženkama AO (Albino Oxford) soja pacova, primenom mikronukleus testa. Eksperimentalne životinje tretirane su intraperitonealno sa tri različite koncentracije kadmijum-hlorida (CdCl$_2$) i to: 0,5, 1 i 2 mg CdCl$_2$ po kg telesne mase. Kontrolne životinje primile su istu količinu sterilnog fiziološkog rastvora sa fosfatnim puferom. Rezultati su pokazali da koncentracija CdCl$_2$ od 2 mg/kg t.m. prouzrokuje statistički visoko značajno (P < 0,001) povećanje produkcije MNA u PHE košane srži, i taj efekat je koncentracijski zavisan. Niže koncentracija CdCl$_2$ koje su korišćene u ovom istraživanju (0,5 i 1 mg/kg t.m.) takođe su prouzrokovale produkciju MNA, ali efakat nije bio statistički visoko značajan. Polne razlike u produkciji MNA u PHE koštene srži, nakon akutnog izlaganja različitim eksperimentalnim koncentracijama CdCl$_2$, nisu uočene u našem istraživanju. Dobijeni rezultati ukazuju na potencijal CdCl$_2$ da prouzrokuje genotoksične efekte u koštanoj srži pacova AO soja i upotpunjuju bazu podataka o genotoksičnosti ovog zagađivača životne sredine, koji je najčešće uzročnik bolesti ljudi izloženih Cd u industrijskoj proizvodnji i onih koji konzumiranju cigarete i hranu kontaminiranu Cd.