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A study on the investigation of cadmium chloride genotoxicity in rat bone marrow using micronucleus test and chromosome aberration analysis

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In this study, we investigated the genotoxic and cytotoxic potential of cadmium chloride ($CdCl_2$) in Wistar rat tibia bone marrow cells, using the structural chromosomal aberration (SCA) and micronucleus (MN) test systems. $CdCl_2$ was administered to adult female rats as repeated i.p. doses of 0.5 mg/kg b.w. for 18 week (four months) at 48 h intervals. Mitomycin C (MMC) was used as a positive control (2 mg/kg b.w.). This study shows that cadmium chloride treatment significantly induced the frequency of micronucleus in polychromatic erythrocytes in tibia bone marrow. This increase in micronucleus frequency shows that cadmium has a genotoxic effect on bone marrow at this level. Also, in order to determine cytotoxicity in bone marrow, the ratio of polychromatic erythrocytes to normochromatic erythrocytes was calculated in bone marrow. The results of this study indicate that $CdCl_2$ decreased this ratio. The decrease of this ratio in bone marrow shows $CdCl_2$ may lead to cytotoxicity. We have reported that 0.5 mg/kg-level chronic exposure to cadmium (Cd) has an injurious effect on bone marrow. Our findings indicate that $CdCl_2$ has a cytotoxic and genotoxic effect on rat bone marrow at chronic exposure. *Toxicology and Industrial Health* 2005; **21:** 243–248.

Key words: *cadmium chloride; micronucleus polychromatic erythrocytes; structural chromosomal aberrations; Wistar rat*

Introduction

Cadmium is a heavy metal and a significant environmental threat owing to its toxicity and ability to accumulate in living organisms (Agency for Toxic Substances and Disease Registry (ATSDR), 1997; Stoeppler, 1991). Recently, the main uses of cadmium are for nickel-cadmium battery manufacture, pigments and plastic stabilizers, whereas applications in alloys, solders and electroplating show a decreasing trend (IARC, 1993). Cadmium poisoning in the environment has become an alarming problem in recent years.

Cadmium compounds that are carcinogenic in some animal species have been classified as a human carcinogen by the International Agency for Research on Cancer (IARC). It causes the formation of carcinogenesis on humans and experimental animals. DNA damage is a critical factor in carcinogenesis (Inami and Mochizuki, 2002).

Cadmium (II) induces DNA lesions in intact cell and affects their repair (Dally and Hartwig, 1997; Rossman *et al*., 1992). It has the ability to

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accumulate in living organism. Cadmium is listed by the US Environmental Protection Agency (EPA) as one of 126 priority pollutants. It is estimated that the half-life of cadmium in humans is between 30 years (Jin *et al*., 1998).

Privezentsev *et al*. (1996) set forth two potential mechanisms of genotoxicity of cadmium: (1) direct Cd–DNA interaction which leads to single strand break in DNA; (2) the action of incision nuclease during repair process in DNA. It is reported that cadmium chloride can interfere with chromatin condensation.

According to data obtained from previous studies, both *in vivo* and *in vitro* induction of chromosome aberration by cadmium compounds were ambiguous. In cytogenetic studies performed on workers exposed occupationally to cadmium; it has also been shown that chromosome aberration was linked to cadmium exposure in some cases. Beyersmann and Hechtenberg (1997) reported that cadmium compounds enhance the genotoxicity of mutagens and inhibit DNA repair processes. Hartman and Hartwig (1998) indicate that cadmium blocks DNA repair by disturbing protein interactions involved in DNA damage recognition. Snow (1992) determined that the high concentrations of cadmium compounds induce DNA strand break and inhibit the biosynthesis of DNA, RNA and proteins. Cadmium may lead to direct genetic damage by interacting with chromatin to form strand break, crosslinks or alteration in DNA. Besides, cadmium may play a role indirectly by expending antioxidant levels and thus increasing intracellular hydrogen peroxide. Increases in levels of hydrogen peroxide of intracellular lead the formation of free radicals that interact with DNA.

Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact chromosome lagging behind in the anaphase stage of cell division (Schmid, 1973). Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis. Micronucleus test has been extensively used for ten years to evaluate genotoxicity of chemical substances and is the most frequently used method in rodent species. The *in vivo* micronucleus assay is one of the most common assay *in vivo* screening tests. It detects clastogens and/or aneugens. The determination of score of micronucleus is less time consuming. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage. (Heddle *et al*., 1983; MacGregor *et al*., 1987; Hayashi *et al.*, 1994; Çelik *et al.*, 2005).

Cadmium is also toxic. Generally, the route and duration of exposure determine ultimately which organ becomes the target of Cd toxicity. Chronic exposure can lead to emphysema, obstructive airway disease, permanent renal dysfunction, bone disorders immunosuppressant (Friberg *et al.*, 1986). In rodents, Cd has been associated with injection site tumors, as well as tumors of the lung, prostate, liver, testes and hematopoietic system (Waalkes *et al*., 1994).

The objective of present study was to evaluate the cytotoxic effects, by calculating the ratio of polychromatic erythrocytes (PCEs) to normochromatic erytrocytes (NCEs), and the genotoxic effects, by using chromosomal aberrations and micronuclei as genetic endpoints, of cadmium chloride in bone marrow in *in vivo* at chronic dose.

Materials and methods

Chemicals

Cadmium chloride ($CdCl_2$ H_2O, Fluka) (CAS No.: 20899) was dissolved in distilled water. Mitomycin C (MMC) (2 mg/kg b.w.) was used as positive control.

Animals and treatment

Healthy adult female Swiss albino rats (Wistar rat) (8–10 weeks of age, with average body weight (b.w.) of 230–250 g) were used in this study. Rats were obtained from the Experimental Animal Center, University of Erciyes, Kayseri, Turkey. The rats were randomly selected and housed in polycarbonate boxes (four rats per box) with steel wire tops and rice husk bedding. They were maintained in a controlled atmosphere with a 12:12 h dark/light cycle, a temperature of $22 \pm 2^{\circ}C$ and 50–70% humidity with free access to pelleted feed and tap water. The animals were supplied with dry food pellets. The animals were allowed to acclimatize for one week before treatment.

Dose selection

$CdCl_2$ was applied to animals intraperitoneally at chronic dose. $CdCl_2$ dose was determined by our prestudy. Firstly, 1 mg/kg b.w. of $CdCl_2$ was given to rats i.p. However, the animals could not tolerate the high dose. Therefore, the dose was halved (0.5 mg/kg b.w.) and the study continued with a dose of 0.5 mg/kg of $CdCl_2$.

MMC (2 mg/kg) was used as a positive control. The positive control and the untreated control rats were identically treated with equal volumes of normal saline only via intraperitoneal (i.p.) injection. It is acceptable that a positive control is administered by a different route from or the same as the test agent and that it is given only a single time (Hayashi *et al*., 1994). MMC was given as a single dose.

Chromosome aberration assay

The rats were sacrificed 24 h after administration of the last treatment for chromosome aberration analysis. Cytogenetic analysis was performed on tibia bone marrow cells according to the recommendations of Adler (1984), with slight modifications. Experimental animals were injected (i.p.) with colchicines (4 mg/kg) 1.5 h before sacrifice. Both tibia were dissected out and cleaned of any adhering muscle. Bone marrow cells were collected from both tibias by flushing in KCl (0.075 M, at 37°C, 5 mL) and incubated at 37°C for 25 min. Material was centrifuged at 2000 rpm for 10 min, fixed in methanol:acetic acid (Carnoy's fixative, 3:1 v/v). Centrifugation and fixation (in the cold) were repeated five times at least at intervals of 20 min. The material was resuspended in a little volume of fixative, dropped onto chilled slides, flame-dried and stained in 5% Sorenson buffered Giemsa (pH: 6.8). At least 75 good metaphases containing 42 chromosomes were examined per animal to score different types of aberrations. To avoid the bias, slides were coded as blind.

Micronucleus test

Rats were sacrificed by cervical dislocation 28 h after the last treatment. The frequency of micronucleated erythrocytes in tibia bone marrow was evaluated according to the procedure of Schmid (1976) as performed in femoral bone marrow, with slight modifications. The bone marrow was flushed out from both tibias using 1 mL fetal bovine serum and centrifuged at 2000 rpm for 10 min. The supernatant was discarded. Evenly spread bone marrow smears were stained using the Feulgen stain. Slides were scored at a magnification of $1000\times$ using a light microscope.

Scoring

In order to determine the frequency of micronucleus 2000 PCEs per animals were scored to calculate the MN frequencies, and 200 erythrocytes (immature and mature cells) were examined to determine the ratio of PCE to normochromatic erythrocytes (NCEs).

Statistical analysis

Data were compared by one-way variance analysis. Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were carried out by least significant difference (LSD) test. $P<0.05$ was considered as the level of significance.

Results

The metaphase analysis of the tibia bone marrow cells shows the different types of chromosomal aberrations including chromatid and isochromatid (chromosome) types of gaps and break, fragment, dicentric chromosome, chromatid gap. Also, the chromatid break was observed to be more frequent than other aberrations. In this study, numbers of numerical chromosome aberrations were not scored because their frequency is very low.

The results showing the frequency of chromosome aberrations and micronucleus, the ratios of PCEs to NCEs are depicted in Tables 1 and 2, respectively. When compared to controls, $CdCl_2$ (0.5 mg/kg) induced the chromosome aberrations frequency and MN frequency in 1000 polychromatic erythrocytes.

The frequency of chromosomal aberrations significantly increased with $CdCl_2$ when compared to control ($P<0.05$). The mean of the induced chro-

Table 1. Frequency of chromosome aberrations in bone marrow cells of female Wistar rat induced by $CdCl_2$.

Group	Total number of examined metaphases (*n*)	Chromatid		Chromosome		Fragment	Dic	TSCA	Mean (%) ± SE
		Gap	Break	Gap	Break				
Isotonic saline	400/4	5	3	–	2	5	–	15	2.5 ± 0.28
$CdCl_2$ (0.5 mg/kg)	400/4	12	14	–	3	7	4	40	7.0 ± 0.40**
PC MMC (2 mg/kg)	400/4	24	20	22	21	18	3	108	27.0 ± 2.12***

n, number of animal; TSCA, total structural chromosome aberrations; Dic, dicentric chromosome; $CdCl_2$, cadmium chloride; MMC, mitomycin C; PC, positive control.
**$P < 0.05$ compared to control.
***$P < 0.001$ compared to control.

mosome aberrations reached 7.00 ± 0.40, while this mean was 2.50 ± 0.28 in control rats.

$CdCl_2$ treatment induced the frequency of MN in rat bone marrow. The mean of MN reached 5.5 ± 0.50, while the frequency of MN was 2.5 ± 0.28 in control rats. $CdCl_2$ (0.5 mg/kg) induced significantly the MN frequency when compared to control ($P < 0.05$).

In addition to this, the ratios of PCE/NCE measured in whole bone marrow samples are summarized in Table 2. $CdCl_2$ treatment decreased the PCE/NCE ratio ($P < 0.001$) when compared with controls.

Discussion

Pollution by heavy metals is an important problem due to their stable and persistent existence in the environment. Cadmium is one of the most toxic environmental and industrial pollutants affecting DNA (Valverde *et al*., 2001). Its ability to accumulate in living organisms is a significant threat to the environment and public health. The genotoxicity and mutagenity, toxicity studies concerning Cd have produced controversial results depending on the assay used, *in vivo* and *in vitro*. In a study performed in Chinese hamster cells, it was reported that cadmium compounds caused chromosome damage (Rozgaj *et al*., 1983) as well as *in vivo* experiment (Han *et al.*, 1992). In this report, cadmium seemed to be a strong clastogen and a cytotoxic agent in rat haemopoietic system. Our results show that Cd induces cytotoxicity and genotoxicity in rat bone marrow at chronic treatment.

Reports on cadmium-induced chromosomal alterations in mammalian cells *in vitro* have been contradictory. In a study performed with human lymphocytes by Kasuba and Rozgaj (2002), $CdCl_2$

Table 2. Micronucleus induction and number of PCEs in $CdCl_2$-exposed rat bone marrow.

Treatment group	Rat No.	MN per 2000 PCEs	PCEs per 200 erythrocytes
Isotonic saline (vehicle control)	1	2	109
	2	3	108
	3	3	111
	4	2	110
Mean ± SE		2.50 ± 0.28	109.5 ± 0.64
$CdCl_2$ (0.5 mg/kg)	1	4	67
	2	6	72
	3	6	75
	4	6	73
Mean ± SE		5.50 ± 0.50**	71.7 ± 1.70**
MMC (PC) (2 mg/kg)	1	22	48
	2	20	46
	3	23	45
	4	25	48
Mean ± SE		22.5 ± 1.04***	46.7 ± 0.75***

$CdCl_2$, cadmium chloride; MMC, mitomycin C; PC, positive control.
**$P < 0.05$ compared to control.
***$P < 0.001$ compared to control.

induced the micronucleus frequency. They found that $CdCl_2$, regardless of concentration, applied in the early S phase produced a significant increase in micronucleus. Wang and Lee (2001) investigated the effect of fetal calf serum on the cadmium clastogenity in Chinese hamster ovary cells. They found that cadmium chloride induced the genotoxicity.

Yamada *et al*. (1993) showed that Cd^{2+} inhibits DNA pre-replicational repair and perhaps DNA excision repair, thereby causing co-clastogenic effects. Inhibition of DNA repair has been recommended to occur when Cd^{2+} competes with Zn^{2+} for a common binding to enzymes involved in DNA synthesis. DNA damage induced by environmental mutagenic is repaired predominantly by nucleotide excision repair (NER). Diverse DNA repair systems have evolved to remove DNA lesions derived from exogenous and endogenous sources. With respect to endogenous DNA damage, DNA replication errors are corrected by mismatch repair system and removed from the newly synthesized DNA strand shortly after DNA replication.

However, much is known about the ability of metallic compounds to induce chromosome aberrations in bone marrow cells of laboratory animals. The data of this study indicated that cadmium chloride is a potent inducer of chromosomal aberration in rat bone marrow. Latinwo *et al*. (1997) showed the increased levels of DNA single-strand breaks in rat kidney cells induced by cadmium chloride given to rats via gavage. In a study performed by Kasuba *et al*. (2002), $CdCl_2$ (0.5 mg/kg) was administered to rats for 9 day and determined the clastogenic activity of $CdCl_2$ at acute treatment. Adler and Ashby (1989) explained that $CdCl_2$ has a clastogenic activity on the rat somatic cells.

According to studies performed with $CdCl_2$, both chronic treatment and in acute treatment at the same dose (0.5 mg/kg) of $CdCl_2$ is genotoxic. Cadmium chloride significantly increased the frequency of micronuclei and chromosome aberration, particularly, single-stand break, in rat polychromatic erythrocytes of bone marrow. In the present study, the increase in DNA single-strand break indicates the interaction between DNA and $CdCl_2$. Additionally, Fahmy and Aly (2000) investigated the effects of $CdCl_2$; they observed that $CdCl_2$ treatment reduced the number of spermatocytes. Misra *et al.* (1998) investigated the cytotoxic effect of $CdCl_2$ in four different rodent cell lines and found that cadmium is not directly genotoxic. In our study, we also determined that $CdCl_2$ decreased the ratio of PCE/NCE. This result shows the cytotoxic effect of $CdCl_2$ in rat bone marrow at chronic treatment. It is clear that $CdCl_2$ causes the depression or bone marrow cell toxicity. A very significant decrease in proportion would be indicative of a cytotoxic effect. Such a decreased ratio is often used as an indicator of bone marrow cytotoxicity or alterations in erythropoiesis. In normal bone marrow, the PCE:NCE ratio is generally around 1:1 (Schmid, 1973). A decrease in the proportion of PCE is used as an indication of mutagen-induced bone marrow cytotoxicity or changes in erythropoiesis (Suzuki *et al.*, 1989).

Our results show the similarity with other studies investigating the genotoxic effects of $CdCl_2$. Our findings support the results of a study performed by Kasuba *et al*. (2002) and as understood two studies, $CdCl_2$ is genotoxic in both the oral and intraperitoneal route and both acute and chronic treatment at the same dose (0.5 mg/kg).

In conclusion, treatment of $CdCl_2$ applied intraperitoneally showed significant genotoxic health effects by micronucleus and chromosome aberration analysis in the presence of very low cadmium at chronic treatment. The *in vivo* micronucleus and chromosome aberration test used in this study was a very sensitive method to evaluate the chromosomal damage in mammalian cells exposed to chemical substances.

According to these results, this level of cadmium chloride (0.5 mg/kg) seems to potentiate genotoxic and cytotoxic effect in rat bone marrow. Our observations may indicate that an environment polluted with cadmium chloride may lead to severe health effects for mammals.

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