



## RESEARCH ARTICLE

### DNA Damage of Lung Cells from Immature Cadmium-Ingsted Mice

Xue-feng Yang<sup>1</sup>, Hai-tang Zhang<sup>1</sup>, Guo-ying Fan, Dong-yang Liu, Ya-ming Ge, Jin-qing Jiang and Zi-liang Wang\*

College of Animal Science, Henan Institute of Science and Technology, Xinxiang, Henan Province, P.R. China, 453003

\*Corresponding author: [wangzl\\_2008@yahoo.com.cn](mailto:wangzl_2008@yahoo.com.cn)

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#### ABSTRACT

The objective of this study was to investigate the effects of cadmium on DNA damage of lung cells in immature animals. Seventy-two immature mice were randomly divided into twelve cadmium-ingested groups including low dose (1/100 LD<sub>50</sub>, 1.87 mg/kg BW), middle dose (1/50 LD<sub>50</sub>, 3.74 mg/kg BW), high dose (1/25 LD<sub>50</sub>, 7.48 mg/kg BW) and control group, and exposed to cadmium chloride for 10, 20 and 30 days, respectively. Mice were sacrificed after cadmium exposure for different time, and lung cells were collected to investigate DNA damage by comet assay. The results showed that comet tailing ratio, tail length, comet length, tail moment, Olive tail moment and damaged grade of lung cells from immature mice increased along with increasing of cadmium exposure dose and time. In low dose group treated for 30 days, there was significance ( $P < 0.05$ ) in comet length or high significance ( $P < 0.01$ ) in other parameters compared with control group or low dose group treated for 10 days. When mice were exposed to cadmium at high dose for 30 days, DNA of lung cells was damaged most seriously. Our results indicate that cadmium can induce DNA damage of lung cells from immature mice in dose-dependent and time-dependent manners, and DNA will be damaged when immature mice exposed to cadmium for long time even at low dose. Meanwhile, comet assay can be considered as a powerful and sensitive biomarker assay in risk assessment of immature animals exposed to cadmium.

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#### INTRODUCTION

Cadmium is a heavy metal with highly toxic and difficult to biodegradation in the environment (Javed, 2012). Now it has become a prevalent environmental contaminant due to its continuous use in industry and existence in much of electronic trash which is one of threatening environmental problems in the world. After cadmium enters human body through food chain or other approaches, it can be accumulated with biologic half-life as long as 15-20 years (Son *et al.*, 2011) and its excretion rate is very low. It greatly threatens the health of human being. Till now, documents have demonstrated that cadmium can not only generate a wide variety of adverse effects on tissues such as lung, brain, liver, kidney, lung, testis and bone (El-Sokkary and Awadalla, 2011; El-Refaiy and Eissa, 2012), but also lead to cellular DNA damage (Kundu *et al.*, 2009; Zhang *et al.*, 2010; Jabeen and Chaudhry, 2011). As a non-essential metal with teratogenic, carcino-

genic, and mutagenic effects on human and animals (Burger, 2008), its bioaccumulation and toxic effects have attracted considerable attention. Moreover, cadmium has been classified as group 1 carcinogen by the International Agency of Research on Cancer (Sarkar *et al.*, 2013).

Lung is the main target tissue of cadmium (Kundu *et al.*, 2009; Oberdörster, 1992) since inhalation is one of the main exposure routes. Therefore, lung toxicities of cadmium have been extensively investigated. It indicated that cadmium fume and its contaminated air could induce shortness of breath, lung edema, pneumonitis (Seidal *et al.*, 1993) and acute respiratory distress syndromes (Barbee Jr and Prince, 1999) especially for workers. Stosic *et al.* (2010) reported that exposure to particulate cadmium and its fumes or experimental intratracheal instillation resulted in pulmonary toxicity. Kundu *et al.* (2009) found that cadmium could induce lung inflammation independent of cell proliferation. Meanwhile, results showed that cadmium exhibited toxicity to lung by decreasing the viability or inducing the dysfunction of individual pulmonary cells (Lag *et al.*,

<sup>1</sup>These authors contributed equally to this work.

2002). In vitro results also demonstrated that cadmium at micro molar dosages could generate significant toxicity to cells (Othumpangat *et al.*, 2005; Badisa *et al.*, 2008). One study showed that immature mice might be more sensitive than mature mice or rats to toxic metals in the environment (Kostial *et al.*, 1978). However, above mentioned studies focus on identifying the toxicity of cadmium to adult animals. Very little information is known about the toxic effects of cadmium on immature animals. Herein, we aim to investigate DNA damage effects of cadmium on lung cells from immature mice using comet assay. Results demonstrate that cadmium can induce DNA damage of lung cells from immature mice in dose-dependent and time-dependent manners. This may provide important information for the comprehensive understanding of the toxicity of cadmium.

## MATERIALS AND METHODS

**Mice:** All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Henan Province, China. Seventy-two Kunming mice (36 each of male and female) weaned at (20±1) d of age were obtained from Centre for Laboratory Animal of Xinxiang Medical University, China. Mice were housed under standard environmental conditions (12 h light and 12 h dark) and allowed free access to water and standard commercial rodent diet.

**Group and cadmium exposure:** After the acclimation for 5 days, mice were randomly divided into twelve groups (3 male and 3 female mice in each group) including low dose (1/100 LD<sub>50</sub>, 1.87 mg/kg BW), middle dose (1/50 LD<sub>50</sub>, 3.74 mg/kg BW), high dose (1/25 LD<sub>50</sub>, 7.48 mg/kg BW) and control group (deionized water, control), and exposed to cadmium chloride for 10, 20 and 30 days, respectively. LD<sub>50</sub>, the median lethal dose of cadmium chloride via oral administration to mice, was 187 mg/kg BW determined in our previous study (Yang *et al.*, 2012).

The three different concentrations of cadmium chloride solution were prepared with deionized water and were administered to mice as freely drinking water. Deionized water was given to the control group in the same manner as the treatment groups. Mice were sacrificed after exposure to cadmium for different time (i.e. 10, 20 and 30 days).

**Comet assay:** Lungs were removed from each animal, washed 3 times with cold PBS and minced with scissors. Tissue pieces were grinded with glass homogenizer and filtered through a mesh with 48 µm pore size to obtain single cells suspensions. The final concentration of single cells suspensions was adjusted to approximately 10<sup>6</sup>/mL in cold PBS for immediate comet assay.

Comet assay (or single cell gel electrophoresis) was performed according to Singh *et al.* (1988) with slight modification. Briefly, 10 µL of single cells suspensions were mixed with 75 µL of 0.5% (w/v) low melting point agarose at 37°C and layered on 0.5% (w/v) normal agarose pre-coated frosted slides. The slides were covered with coverslips and placed at 4°C for 10 min to allow the agarose to solidify. After coverslips were carefully

removed, and the slides were covered with a layer of low melting point agarose and placed at 4°C for 10 min again. Then the slides were immersed in a freshly prepared alkaline lysis solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, 1% v/v N-lauroyl sarcosine sodium salt, 10% DMSO, and 1% v/v Triton X-100, pH 10) at 4°C for 1 h. After lysing, slides were incubated for 20 min in a horizontal electrophoresis tank side by side with alkaline electrophoresis buffer (0.3 M NaOH and 1 mM Na<sub>2</sub>EDTA, pH 13) at 4°C for DNA unwinding and alkali labile damage expression before electrophoresis. Then samples were electrophoresed for 20 min at 25 V, 300 mA at the same temperature. Following removal of slides from lysis solution, slides were dried with filter paper and neutralized 3 times for 5 min each using neutralization buffer (Tris-HCl 0.4 M, pH 7.5). Subsequently cells were stained with ethidium bromide (30 µg/mL) for at least 20 min and slides were covered with cover slips and stored in a dark humidified chamber until analysis. All procedures were performed under dimmed light to prevent additional DNA damage.

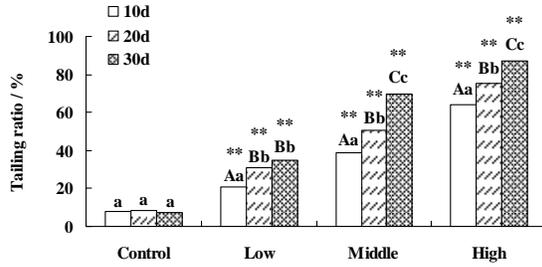
Cells were observed at a magnification of 200× by a fluorescence microscope (Nikon, Japan) with green light excitation and a 590 nm barrier filter. Tailing ratio was calculated by randomly counting tailing DNA in 100 cells/animal. 150 cells per group for each cadmium exposure time point (6 slides of 25 cells/animal) were randomly selected and photographed to measure comet parameters such as tail length (TL, pixels), comet length (CL, pixels), tail moment (TM, arbitrary units), Olive tail moment (OTM, arbitrary units) and amount of DNA in the comet tail (Tail DNA%) by Comet Assay Software Project (version 1.2.3b1). Classification of comets was five damage levels according to Tail DNA%, including grade 0, 1, 2, 3 and 4. Grade 0 was defined as no DNA damage and grade 4 was the most serious DNA damage (Prieto González *et al.*, 2011).

**Statistical analysis:** Tailing ratio among the various groups was freely compared using chi square ( $\chi^2$ ) tests in SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). The data of TL, CL, TM, and OTM were expressed as mean±SEM and freely compared for significance using the Student's t-test in SPSS 10.0. Significance was accepted as P<0.05.

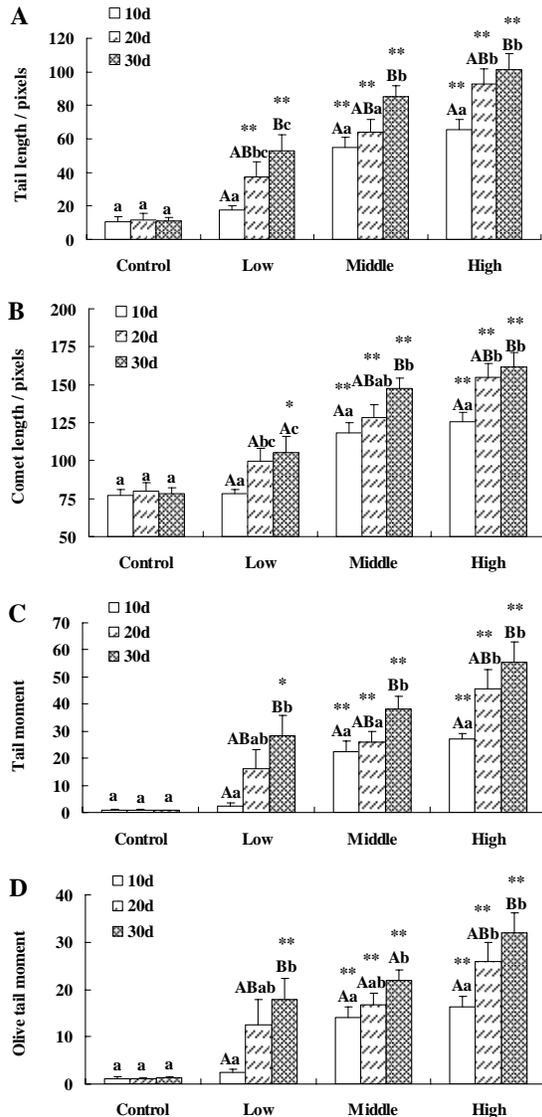
## RESULTS

**Tailing ratio:** As shown in Fig. 1, tailing ratios of lung cells from cadmium treatment groups significantly increased compared to those of control group in a dose-dependent manner (P<0.01). Meanwhile, tailing ratio for cadmium treatment groups increased along with elongation of the exposure time. In other words, lung cellular DNA from immature mice was damaged most seriously when treated with cadmium at high dose for 30 days.

**Comet parameter:** TL and CL are two important parameters of DNA damage in comet assay. The comet assay showed that TL and CL in all treatment groups increased in dose-dependent manners as compared to controls (Fig. 2A and Fig. 2B), but there was no significance between control group and the treatment group at low dose for 10 days (P>0.05). Both parameters



**Fig. 1:** Effect of cadmium on tailing ratio of lung cellular DNA in immature mice. At the same exposure dose levels compared between two treatment groups freely, same letters above the columns indicate  $P > 0.05$ ; different lowercase and capital letters indicate  $P < 0.05$  and  $P < 0.01$ , respectively. Double asterisk above the columns represent  $P < 0.01$  compared to the corresponding control at the same exposure time.



**Fig. 2:** Effect of cadmium on comet parameter of lung cellular DNA in immature mice. Data are shown as mean  $\pm$  SEM for each group of mice ( $n=150$ ). Compared between two treatment groups freely at the same exposure dose levels, same letters above the columns indicate  $P > 0.05$ , different lowercase and capital letters indicate  $P < 0.05$  and  $P < 0.01$ , respectively. Single and double asterisk above the columns represent  $P < 0.05$  and  $P < 0.01$  compared to the corresponding control at the same exposure time, respectively.

also increased in time-dependent manners for every treatment group. In middle and high dose groups treated for 30 days the differences were all highly significant ( $P < 0.01$ ) as compared to those of group treated for 10 days.

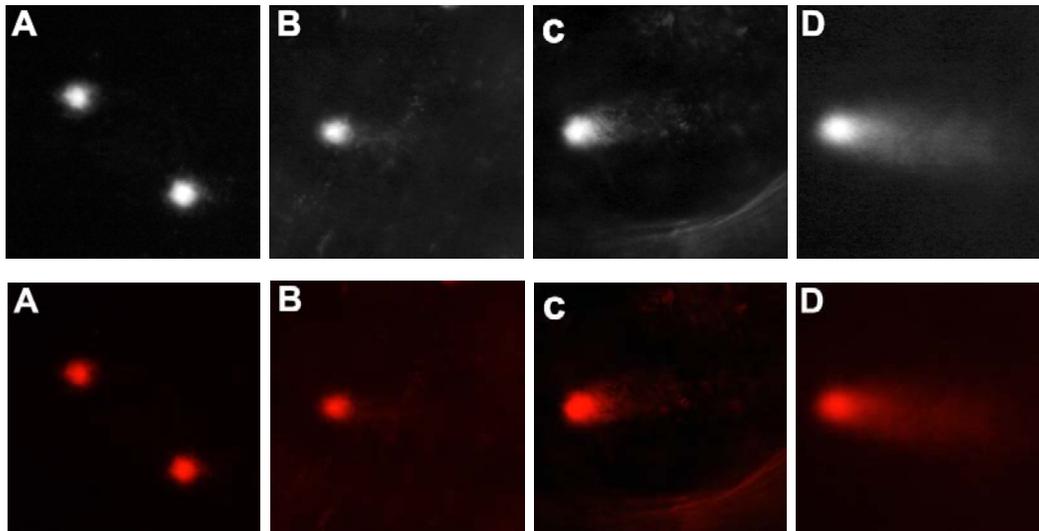
TM and OTM also are commonly used markers of DNA damage in comet assay. As shown in Fig. 2C and Figure 2D, TM and OTM significantly increased along with increasing of cadmium-ingested dose and time. In comparison to control groups, TM and OTM significantly increased ( $P < 0.01$ ) in middle and high dose groups. In every cadmium treatment group, both parameters also increased with elongation of treatment time, and the increasing was highly significant ( $P < 0.01$ ) in high dose group treated for 30 days compared with 10 days.

**Comet images and grade of DNA damage:** The more serious cells were damaged, the longer their tailings were in the comet assay. The typical comet configurations were seen in Figure 3. The percentages of each grade of DNA damage induced by cadmium were shown in Table 1. At exposure time for 10, 20 and 30 days, cells in grade 3 of DNA damage in low dose group were about 1, 2 and 9%, in middle dose group were 8, 12 and 20% and in high dose group were 15, 18 and 29%, respectively. But the most serious grade 4 of DNA damage was not observed in all groups.

## DISCUSSION

Ingestion may be the main pathway for non-occupational exposure of people to cadmium and its compounds while inhalation as the main pathway for occupational exposure. However, subcutaneous (Jabeen and Chaudhry, 2011) or intraperitoneal injection (Kundu *et al.*, 2009) is often selected in most of studies. Data of these studies cannot provide accurate information of the toxicity of cadmium. Therefore, in our study, animals were exposed to cadmium by oral administration with cadmium-containing water for different time, which mimicked real condition of humans exposed to cadmium.

A few studies indicate that younger animals absorb more cadmium in the gastrointestinal tract than adults. Sasser and Jarboe (1980) have proved that absorption of cadmium in the gastrointestinal tract of young guinea pigs was 20-fold higher than that of adult guinea pigs. Kostial *et al.* (1978) reported that the highest oral toxicity was found in the youngest group of rats as indicated by the lowest  $LD_{50}$  values for oral cadmium, which suggested young animals were more sensitive to cadmium than adults. On the other hand, it is well known that cadmium is a cumulative toxin, the biologic half-life in human body is extremely long (as long as 15-20 years) while the excretion rate is low. Therefore, adverse effects of long-term cadmium ingestion even in low amounts should not be ignored especially in immature organisms since immature stage plays critical roles in the mammalian ontogeny development. However, fewer attentions are paid to this special cadmium-ingested stage, and respiratory effects of cadmium on immature animals or human are poorly understood. In the present study, immature mice were used as model animals of cadmium-ingested exposure.



**Fig. 3:** Typical comet images of lung cellular DNA in high dose cadmium-ingested for 30 days group. A: grade 0 of DNA damage, no damage,  $\times 200$ ; B: grade 1 of DNA damage, low damage,  $\times 200$ ; C: grade 2 of DNA damage, medium damage,  $\times 200$ ; D: grade 3 of DNA damage, high damage,  $\times 200$ .

**Table 1 :** Effects of cadmium on grade of lung cells DNA damage from immature mice

Groups	10 d					20 d					30 d				
	G0	G1	G2	G3	G4	G0	G1	G2	G3	G4	G0	G1	G2	G3	G4
Control	98.0	2.0	0	0	0	98.7	1.3	0	0	0	95.3	4.7	0	0	0
Low	92.1	5.3	1.3	1.3	0	82.0	12.0	4.0	2.0	0	78.6	10.0	2.7	8.7	0
Middle	73.3	8.7	10.0	8.0	0	60.6	16.7	10.7	12.0	0	45.3	14.7	20.0	20.0	0
High	42.0	24.0	18.7	15.3	0	35.3	30.7	16.0	18.0	0	24.7	29.3	16.7	29.3	0

G0=Grade 0, G1=Grade 1, G2=Grade 2, G3=Grade 3, G4=Grade 4.

The comet assay is a simple, sensitive, reliable and rapid method that can be used to microscopically detect DNA damage induced by environmental chemical substance at the individual cell, tissues and organs level. Recently, some studies have introduced advantages of this assay in genotoxicological, ecotoxicological and biomonitoring research fields (Kumaravel *et al.*, 2009; Baumgartner *et al.*, 2009; Fikrová *et al.*, 2011). It has been widely used for the sensitive assessment of DNA damage in occupationally and environmentally exposed population. In our study, comet assay was carried out to investigate lung cells DNA damage in immature cadmium-ingested mice.

DNA is the hereditary material in living cell. DNA integrity is subject to damage from various chemicals and environmental risk agents such as heavy metal, radiation, pesticides and so on. Several studies have proven that cadmium can induce DNA-protein cross-links, DNA strand breaks, oxidative DNA damage and DNA repair inhibition etc (Pei and Xu, 2003; Yousef *et al.*, 2010; Jia *et al.*, 2011). Cadmium can also inhibit DNA polymerase activity and reduce synthetic DNA and high-fidelity of DNA polymerase in base pairing (Palus *et al.*, 2003). Potts *et al.* (2001) reported cadmium could interfere with antioxidative function of alveolar epithelial cells, and then injure cellular DNA and inhibit DNA repair. Our results showed that comet tailing ratio, TL, CL, TM, OTM and damaged grade of lung cells from immature cadmium-ingested mice increased along with increasing of cadmium-ingested dose and time, and when mice were exposed to cadmium at high dose for 30 days, DNA of lung cells was damaged most seriously. As for low dose group treated for 30 days, there was significance ( $P < 0.05$ )

in CL or high significance ( $P < 0.01$ ) in other parameters compared with control group or low dose group treated for 10 days. In other words, DNA will be damaged when immature mice exposed to cadmium for long time even at low dose. Kundu *et al.* (2009) found that cadmium exposure with intraperitoneal injection at low dose (5 mg/kg BW) showed no significant DNA damage to Swiss albino mice (average weight of 25 g), but high dose (80 mg/kg BW) induced comet tail formation. In consideration of the age of animals, this result may be consistent with our data. We mainly considered that lung cells of immature animals may be more sensitive to cadmium exposure than those of adult animals. The nuclei of lung cells from control mice mainly maintained normal circular form (Figure 3A), with little or no comet formation, and almost no DNA damage in cells. In contrast, lung cells from mice treated with cadmium at different doses for different time showed tail formation and cellular deformity (Figure 3B-3D).

**Conclusion:** Our results indicate that cadmium can induce DNA damage of lung cells from immature mice in dose-dependent and time-dependent manners. Meanwhile, comet assay can be considered as a powerful and sensitive biomarker assay in risk assessment of immature animals exposed to environmental cadmium pollutants.

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