Gender Differences in Pulmonary Inflammation Following Systemic Cadmium Administration in Rats

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Objective To examine the presence of gender differences in pulmonary inflammation evoked by acute systemic cadmium administration in rats. **Methods** Presence of basic indicators of lung inflammation (inflammatory cytokine lung content, leukocyte infiltration and activity of cells recovered from lungs by enzyme digestion) was analyzed and compared in animals of the two sexes. **Results** Intraperitoneal administration of cadmium (1.0 mg/kg) resulted in higher cadmium content in lungs of female rats. Higher tumor necrosis factor (TNF) content was noted in lung homogenates of male rats, while interleukin-6 (IL-6) content was slightly, but signifaicantly greater in lungs of female rats. Increased leukocyte infiltration was observed in lungs of male rats, mainly due to neutrophils. Increased responsiveness to phorbol myristate acetate (PMA) stimulation was noted in cells recovered from lungs of male rats. Rise in intracellular content of myeloperoxidase (MPO) was noted in lung cells from cadmium-treated rats of both sexes, but higher in cells from male rats. **Conclusion** Presented data documented a more intense pulmonary inflammatory response to systemic cadmium administration in males, with higher IL-6 levels in lungs of female individuals. These sex differences in proinflamatory activity of cadmium in lungs should be taken into consideration in studying the remote toxicity of this heavy metal.

Key words: Rats; Sex; Intraperitoneal cadmium; Pulmonary inflammation

INTRODUCTION

Cadmium is one of the most toxic metals in the environment. Its toxicity as an industrial pollutant, a food contaminant and one of the main components in cigarette smoke is well documented[1]. Cadmium adversely affects a number of organs and tissues including the kidneys, liver, lungs, testis, brain, bone, and blood[2-3]. The liver and kidneys are two primary organs in which the systemic toxicity of this metal is expressed. Exposure to particulate cadmium and cadmium fumes or experimental intratracheal instillation of cadmium results in pulmonary (proximal) toxicity. Perfused or orally administered cadmium caused tissue damage in the gastrointestinal tract.

Toxicity of cadmium to liver and kidneys is the most frequently studied aspect of distal toxicity of this metal. Studies of intraperitoneal cadmium administration in rats demonstrated oxidative stress responses in other organs beside liver, including lungs and brain, suggesting these organs as remote targets of systemic cadmium toxicity[4].

Physiological host parameters (genetic backg round, sex, and age) influence cadmium toxicity[5]. Differential species-[6-7] and strain-dependent[8-9] as well as sex-related[10] sensitivity of liver to cadmium was observed. Differences in tissue disposition of cadmium[7,10] most likely due to hormone-related influences[11-12] were considered relevant for gender differences in cadmium toxicity. Beside tissue distribution, hematological/immunological system was proposed as responsible for differences in cadmium toxicity. The relevance of inflamma tion-related activity to strain[9] and age-related[6,13] differences in hepatotoxicity were reported. Differences observed in systemic inflammatory response to cadmium administration in male and female rats[14] imply the relevance of cadmium-induced inflammatory/immune activity for gender-

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related differences to toxicity of this metal.

We have shown recently that intraperitoneal administration of cadmium to rats was associated with pulmonary inflammation, revealing the lungs as remote inflammatory target of systemic cadmium administration[15]. Having in mind reported gender differences in systemic inflammation in rats[14], the aim of the present study was to investigate whether there are sex-related differences in remote (lung) inflammation of systemically administred cadmium in rats. To this aim, basic indicators of lung inflammation (lung inflammatory cytokine content, leukocyte infiltration and activity) were examined in rats of both sexes. Evidence was obtained to demonstrate the presence of a more vigorous pulmonary inflammation in male rats compared to female ones, but with higher IL-6 levels in lungs of female individuals.

MATERIALS AND METHODS

Chemicals

Cadmium chloride (Serva, Feinbiochemica, Germany) was prepared in sterile pyrogen-free saline. Phenylmethanesulfonyl fluoride (PMSF), hexadecyltrimethylammoniumbromide (HTAB), *o*-dianisidine dihydrochloride, myeloperoxidase (MPO), phorbol-12-myristate 13-acetate (PMA), deoxyribonuclease I (DNase I), 2-thiobarbituric acid and malondialdehyde (MDA), were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Collagenase (type IV) was purchased from Worthington Biochemical Corporation (Lakewood, USA). Coomassie brilliant blue G-250 was purchased from Biorad (CA, USA). PMSF was dissolved in isopropyl alcohol and diluted in sodium phosphate buffer. PMA was dissolved in dimethylsulfoxide (DMSO) at 1 000 greater concentration and diluted before use in cell culture medium. Myeloperoxidase was used dissolved in water. Nitroblue tetrazolium, NBT (ICN Pharmaceutical, Costa Mesa, CA, USA) was dissolved in water to a final concentration of 5 mg/mL. All solutions for cell culture experiments were either prepared under sterile conditions or were sterile filtered (Flowpore, pore size 0.22 μm) before use. Culture medium RPMI-1640 (Flow, ICN Pharmaceuticals) supplemented with 2 mmol/L glutamine, 20 μg/mL gentamycine (Galenika a.d., Serbia), 5% (v/v) heat inactivated fetal calf serum (PAA Laboratories, Austria) was used in cell culture experiments.

Animals and Treatment

Animal treatment was carried out in strict adherence to the guidelines of the Ethical Committee of the Institute for Biological Research "Siniša Stanković" (IBISS), Belgrade, Serbia. Male and female Dark Agouti (DA) rats, conventionally housed at IBISS, 10-12 weeks old, were used. Four to six animals were assigned to each treatment group. Cadmium chloride (Serva, Feinbiochemica, Germany) prepared in sterile pyrogen-free saline was administered *i.p.* in a dosing volume of 0.5 and in concentration calculated so that the animals received 1 mg of cadmium/kg body weight (b.w.). Pyrogen-free saline alone was administered to control animals. All measurements were carried out 24 h following cadmium administration, in animals anesthetized by sodium thiopentone (40 mg/kg b.w. *i.p.*).

Pulmonary Cadmium Determination

Cadmium lung content was determined by atomic absorption spectrometry (AAS; SpectrAA-50, Varian, Inc., Palo Alto, CA, USA) after ashing the lyophilized samples at 550 °C and dissolving the ash in 1mol/L HCl. The concentrations were expressed as nmol Cd/g of tissue wet weight ±SD.

Lung Tissue Myeloperoxidase (MPO) Activity

Myeloperoxidase (MPO) activity was measured in homogenates of lung tissue. After sacrifice, the lungs were removed, cleared of blood and homogenized on ice in phosphate buffered saline containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF). To one volume of crude homogenate two volumes of three times concentrated hexadecyltrimethylammonium bromide (HTAB) in potassium phosphate buffer (50 mmol/L, pH 6.5) was added to achieve final concentration of 0.5% HTAB. Homogenates were subjected to three cycles of freezing and thawing, sonicated for 15-20 s (Bandelin Electronic, UW 2070, Berlin, Germany) at 50% of maximum intensity amplitude and centrifuged. MPO was evaluated by addition of 33 μL of homogenate supernatant to 967 μL of substrate solution (0.147 mg/mL *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mmol/L potassium phosphate buffer, pH 6.0). Absorbance was read at 450 nm after 10 min against the standard of myeloperoxidase. Values were expressed as MPO units/g lung tissue.

Lung Cytokine Content

Tumor necrosis factor (TNF) and interleukin-6 (IL-6) content in lung tissue homogenates were evaluated using enzyme-linked immunosorbent assays (ELISA) for rat TNF (eBioscience Inc., San Diego, CA, USA) and rat IL-6 (R&D systems, Minneapolis, USA) according to manufacturer's instructions. Cytokine titre was calculated by reference to a standard curve constructed with known

amounts of recombinant TNF or IL-6.

Pulmonary Cell Preparation

Lung leukocytes were obtained by collagenase/DNAse-digestion. Lungs were finely minced and incubated with gentle mixing (magnetic stirer) for half an hour in culture medium supplemented with 1.0 mg/mL collagenase and 30 μg/mL DNase at 37 °C. Cells were resuspended in complete medium and counted by improved Neubauer hemocytometer. Cell viability exceeded 95% as determined by trypan blue exclusion. For lung cell composition determination, cytospin preparations of lung cells were prepared and stained with May Grünwald-Giemsa protocol. Differential cell counts were determined by differentiating at least 300 cells from air-dried cytospin preparations.

Pulmonary Cell Activity Assays

Pulmonary cells obtained by collagenase/DNase lung tissue digestion were resuspended in complete culture medium and plated in 96-well microtiter plates. The activation of lung cells was evaluated by the quantitative cytochemical assay for the respiratory burst[16], based upon cell's spontaneous or PMA stimulated capacity to reduce tetrazolium salt nitroblue tetrazolium, NBT. Briefly, NBT (10 μL, 5 mg/mL) was added to the lung cell cultures (5×10^5 cells per well in 50 μL) and incubated for 30 min in the absence (spontaneous NBT reduction) or presence of PMA in dose of 100 ng/mL (stimulated NBT reduction). Formazan produced by cells was extracted overnight in SDS-Cl and absorbance measured at 540/650 nm.

Lung cell myeloperoxidase activity was assessed on the basis of the oxidation of *o*-dianisidine dihydrochloride by cells lysates as described[17]. Apsorbance was read at 450 nm at three-minute intervals up to ten minutes against the standard of myeloperoxidase. Values were expressed as MPO units per 10^6 cells.

Data Display and Statistical Analysis

Results were expressed as mean values of four to six animals±standard deviation (SD). Statistical analysis was performed by using STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA). Statistical significance was defined by Mann-Whitney *U*-test. *P*-values less than 0.05 were considered significant.

RESULTS

Cadmium Lung Content

Intraperitoneal administration of cadmium chloride to rats resulted in increase in cadmium content in lungs of both male and female rats, but statistically higher in females compared to males (Fig. 1). No statistical difference was noted between absolute mass of lungs from males (1.31±0.13 g and 1.27±0.15 g in controls and following cadmium administration, respectivelly) or females (1.06±0.11 g and 1.02±0.08 g in controls and cadmium-administered animals, respectivelly).

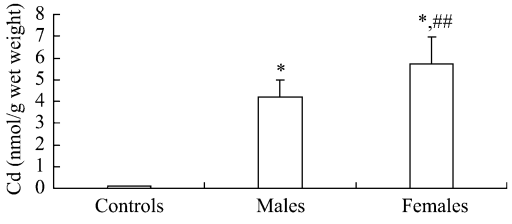


FIG. 1. Cadmium lung content following *ip*. administration. Results are expressed as the mean±SD of four to six rats per group. *denotes $P<0.05$ between cadmium and control groups, ##denotes $P<0.01$ between males and females.

Lung Cytokine Content Following Cadmium Admistration

Evaluation of inflammatory cytokine content in lung homogenates of cadmium-treated rats revealed increase in TNF in male rats (Fig. 2A). In contrast, increased IL-6 content was measured in lung homogenates in female rats (Fig. 2B).

Lung Cell Infiltration Following Cadmium Administration

Administration of cadmium resulted in increased numbers of total leukocytes recovered by enzyme digestion from lungs of male rats compared to control rats (Fig. 3A). In contrast, no increase in numbers of cells recovered from lungs of cadmium-treated female rats was noted (Fig. 3B). Differential cell counting revealed increase in numbers of neutrophils in male rats compared to control and cadmium-treated female rats. Determination of myeloperoxidase (MPO) content in lung homogenates corroborated neutrophil infiltration in lungs of male rats (Fig. 3C).

Lung Cell Activity

Cadmium administration to male rats resulted in increased PMA-stimulated capacity of lung cells to reduce NBT compared to lung cels from control rats (Fig. 4A). No changes in this activity was noted in cultures of lung cells from cadmium-treated female rats (Fig . 4B).

Increased intracellular myeloperoxidase (MPO) content was noted in lung cells from both male and

female cadmium-treated rats (Fig. 5). MPO content was, however, significantly higher in male rats than in female ones injected with cadmium.

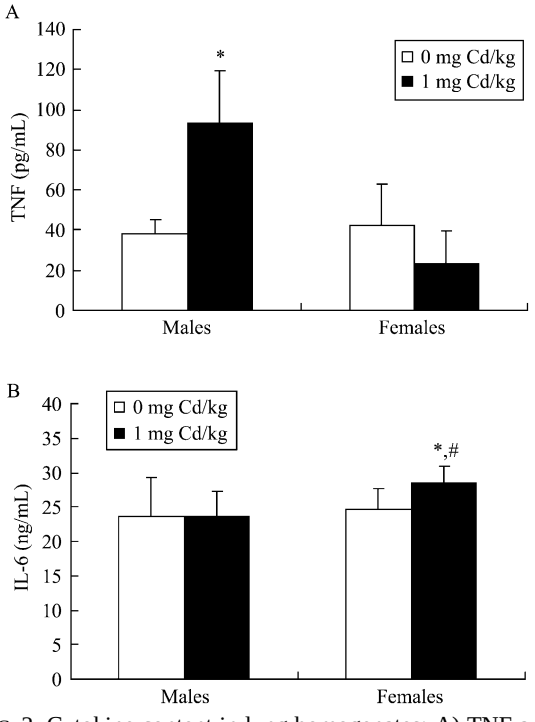
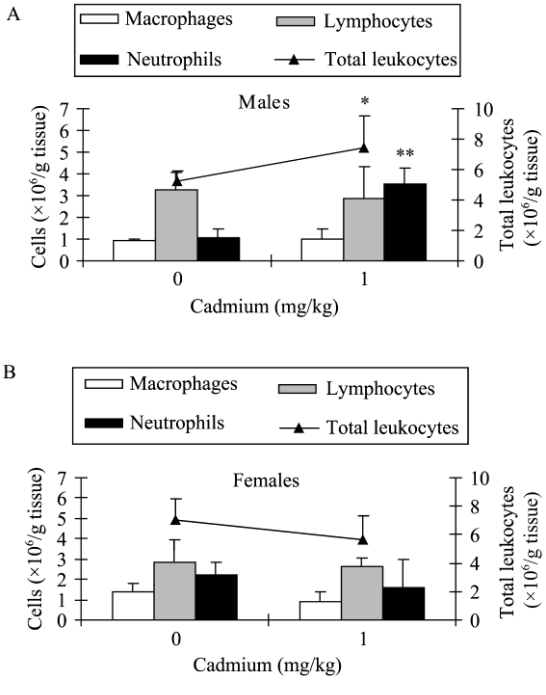


FIG. 2. Cytokine content in lung homogenates: A) TNF and B) IL-6. Results are expressed as the mean ±SD of four to six rats per group. *denotes $P<0.05$ between cadmium and control groups, #denotes $P<0.05$ between males and females.



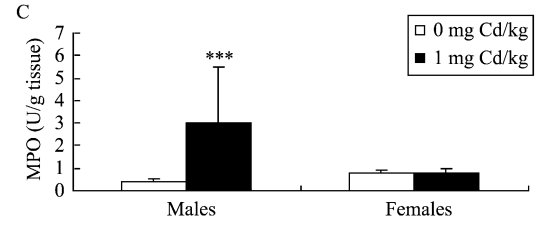


FIG. 3. Total and differential counts of cells recovered from lungs by collagenase digestion in A) males and B) females rats, C) Myeloperoxidese (MPO) content in lungs following Cd administration. Results are expressed as the mean ±SD of four to six rats per group. *, **, and *** denotes $P<0.05$, $P<0.01$ and $P<0.001$, respectively between cadmium and control groups.

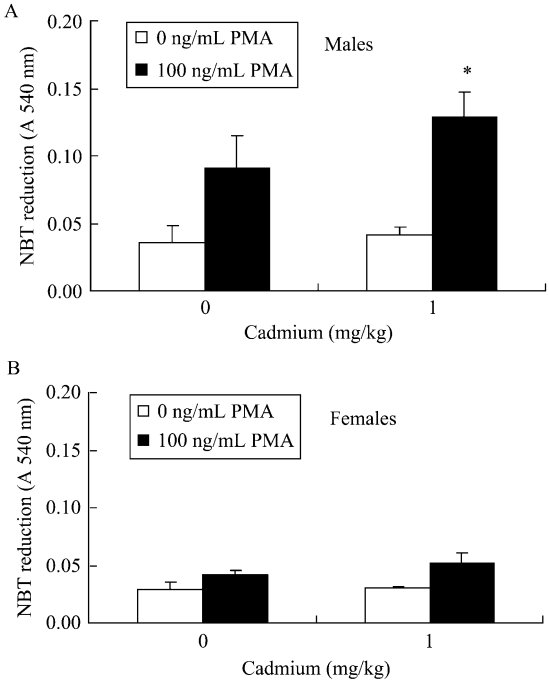


FIG. 4. Spontaneous and PMA-stimulated NBT reducing activity in A) males and B) females rats. Results are expressed as the mean ±SD of four to six rats per group. *denotes $P<0.05$ between cadmium and control groups.

DISCUSSION

In this study, sex differences in pulmonary inflammatory response following systemic cadmium administration were evaluated by measuring biochemical (cytokines) and cellular parametars of inflammation.

Increase in cadmium lung content in males is consistent with our previous study, showing an increase in pulmonary cadmium accumulation in DA rats of this sex[15]. Higher cadmium lung content in

female rats might possibly be ascribed to a greater absorption of cadmium noted in them following 3-day exposure to this metal[10]. It is in broad concordance with the data which showed greater retention of this heavy metal in women compared to men[18].

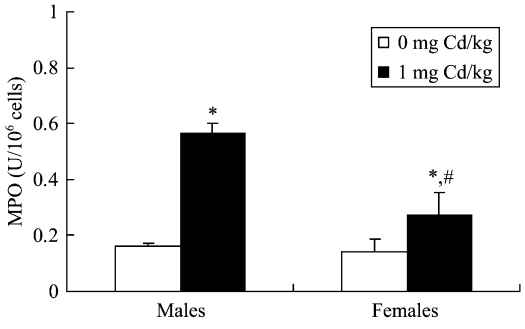


FIG. 5. Lung cell myeloperoxidase (MPO) intracellular content. Results are expressed as the mean ±SD of four to six rats per group. *denotes $P<0.05$ between cadmium and control groups, #denotes $P<0.05$ between males and females.

Increased pulmonary levels of TNF noted in males might have resulted from increased macrophage cell production as reported in rats administered intratracheally with cadmium[19]. Higher levels of TNF in homogenates of lungs from male animals is in concordance with data which showed greater production of TNF by cells in bronchoalveolar lavage fluid in mice with LPS-induced airway inflammation[20]. Slightly higher content of IL-6 in homogenates of lungs from female rats imply higher production of this cytokine by lung cells. It might, possibly, be associted with cadmium-induced production of IL-6 by resident cells, in line with the data which showed increased IL-6 production by splenic macrophages in female mice in settings of tissue injury[21-22]. Higher IL-6 content in lungs of female rats might have contributed to low/unchanged levels of TNF, as IL-6 was shown to act as antiinflammatory cytokine which regulates the levels of inflammatory cytokines in acute inflammation in mice[23].

Increased infiltration of leukocytes to lungs of male rats intoxicated with cadmium is in accordance with data which demonstrated higher accumulation of leukocytes in alveolar spaces of male mice in settings of acute pulmonary response to inflammatory stimulus lipopolysaccharide[20,24]. Increased numbers of polymorphonuclear leukocytes accounted for high total leukocyte numbers in lungs of cadmium-treated rats, in line with increased accumulation of polymorphonuclear leukocytes noted in male mice in LPS-induced pulmonary inflammation[24] and exposed to cigarette smoke[25]. Increased respiratory burst (NBT reducing capacity) to egzogenous stimulation with PMA in males, imply «priming», i.e. the state in which the functional responses to an activating stimulus are potentiated/amplified by a prior exposure to a priming stimuli[26]. Differences in signalling pathways involved in phagocyte cell activity might have contributed to higher propensity of phagocytes from male rats administered with cadmium to respond to exogenous stimuli as shown for higher effector phagocyte activity in male rats in settings of tissue trauma-induced inflammation[27]. Increased inflammatory mediators detected in systemic microenvironment of male rats intoxicated with cadmium[14] might have contributed to differential lung cell response as well.

Increased intracellular levels of myeloperoxidase (MPO) noted in lung cells from animals of both sexes is in line with the potential of heavy metals to stimulate this aspect of activity of neutrophils[28]. It could be ascribed to neutrophils, a common main source of MPO activity[29]. However, activated macrophages might also be a signifiant source of MPO as shown in mice[30]. Higher levels of MPO in polymorphonuclear leukocytes from cadmium-treated male animals might have accounted for increased levels of MPO in lung homogenates of male rats in our study as shown in acute LPS-induced pulmonary inflammation in mice[24].

Observed gender differences in pulmonary responses to systemic cadmium administration might be ascribed to hormonal influences known to influence inflammatory responses in animals[31]. Lower levels of TNF as well as lower magnitude of pulmonary leukocyte infiltration and cell activity noted in females might possibly be ascribed to a suppressive role of female hormones as shown for ovarial hormones in various settings fo inflammation[32-34]. However, higher levels of IL-6 in lungs of females administered with cadmium, suggest that inflammation might have changed female sex steroid-dependent cytokine regulation as shown in trauma/hemorrhage in mice[22].

Activation of phagocytes in lungs of male rats administered intraperitoneally with cadmium, represented a potential risk for tissue injury. Owing to the production of a variety of effector molecules including proteolytic enzymes, reactive oxygen species and cytokines, some of them with the potential of tissue injury, both inflammatory macrophages[35] and neutrophils[36] might be deleterious when excessively activated. Increased intracellular lung cell content of myeloperoxidase noted in males and females represented a risk for tissue injury as well. This heme-contaning enzyme generated highly reactive species such as hypochlorous acid[37] which was shown to induce endothelial cell death and

desquamation *in vitro*[38]. Generation of hypochlorous acid and tyrosyl radical by MPO was suggested as underlying mechanism of oxidative damage of artery wall[39]. Negligible (NBT reduction) or lower levels (intracellular MPO) of lung cell activity in female rats imply that females are less prone to cadmium-induced pulmonary cell injury. This is in line with studies which demonstrated resistance of female rats to shock[32-33] and carageenan-induced inflammatory lung injury[34].

In conclusion, the presented data show that cadmium administration to rats induced pulmonary inflammation that was more intense in males than in females. The biological implications of such differential response warrant future investigation.

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