Gender Differences in Acute Cadmium-Induced Systemic Inflammation in Rats[1]

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Objective To examine the presence of gender differences in pro-inflammatory potential of cadmium in rats by comparing systemic inflammatory response to acute cadmium intoxication in animals of the two sexes. **Methods** Basic aspects of this response were evaluated, including plasma levels of inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) and of major rat acute phase protein alpha 2-macroglobulin (alpha2-M), as soluble indicators of inflammation, and the number and activity of peripheral blood leukocytes, as cellular indicators of inflammation. **Results** Differential increases of IL-6 and alpha 2-M (higher in males than in females) in peripheral blood cell counts and types (leukocytosis and shift in the ratio of granulocytes to lymphocytes more pronounced in males *vs* females) and in levels of neutrophil priming (higher in males *vs* females) were noted. **Conclusion** The data document a more intense inflammatory response to cadmium administration in males. The sex differences in inflammatory effects of cadmium might be taken into consideration in studying the toxicity of this heavy metal.

Key words: Cadmium; Sex; Rats; Inflammation

INTRODUCTION

Cadmium is one of the most toxic metals in our environment. As an industrial pollutant, a food contaminant and one of the main components in cigarette smoke, its toxicity is well known[1]. Cadmium adversely affects a number of organs and tissues including kidney, liver, lung, testis, brain, bone and blood[2-3]. Extensive studies have been carried out to identify the mechanisms of cadmum toxicity in terms of target tissue damage/dysfunction. Two interrelated pathways seem to be involved in acute toxicity to the liver[4]. Although cadmium does not have the ability to directly generate free radicals[5], oxidative damage of polyunsaturated fatty acids of membrane phospholipids is considered to be a primary mechanism of cadmium hepatotoxicity and its toxicity to other organs[6-7]. The second pathway of toxicity is related to the inflammation evoked by focal cadmium-induced tissue damage, including an influx of leukocytes into the tissue and their activation[8-10] and by amplifying circuits in the course of tissue inflammatory response[8,11].

Cadmium toxicity is determined by intrinsic physiological host parameters including genetic background, sex and age[12]. Differences in hepatotoxicity have been noted in rats of various strains[13-14] and different sex[15], as well as in rats[16] and mice[17] of different ages. Differential sensitivity to cadmium has been explained by different levels of tissue/organ cadmium disposition[15,17] and/or methallothionein tissue content[17]. Hormone-dependent cadmium tissue distribution is considered relevant to gender differences in cadmium toxicity[18-19]. However, comparison between cadmium accumulation in various organs of animals of the same strain[16] and in same organs of animals of different sex[15], along with assessment of the relationship between organ cadmium burden and cadmium effects in animals of different strain[14], suggests that cadmium toxicity cannot be explained simply by differences in cadmium distribution and/or metallothionein induction. It has been proposed by some authors that other physiological homeostatic

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systems, principally the hematological/immunological system, might account for differences in cadmium toxicity[14]. Recent investigations have demonstrated the relevance of inflammation-related components to strain-[14] and age-related differences in hepatotoxicity[16,20]. Nonetheless, while these studies suggest the significance of cadmium-induced inflammation for host parameter-related differential sensitivity to cadmium, little is known about the effects of gender on the inflammatory potential of cadmium.

Our previous studies have shown the presence of an acute systemic inflammatory response to *i.p.* cadmium administration in male rats[21-22]. Induction of this response at cadmium doses that induced hepatotoxicity and nephrotoxicity, along with the remote (lung) toxicity, pointed out this less frequently discussed aspect of acute cadmium toxicity. By using this model, gender differences in inflammatory response to acute cadmium intoxication in rats were examined in this study. To determine the cadmium dose to be subsequently employed in the study, the toxicity of cadmium to liver and kidneys was evaluated first in animals of both sexes. Then, soluble and cellular indicators of inflammation were determined in male and female rats. The former included plasma levels of inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) and of the principal acute phase protein in the rat, alpha$_2$-macroglobulin (α_2M)[23]. The latter included quantitative and qualitative changes in peripheral blood leukocytes, including polymorphonuclear (PMN) leukocyte priming. Evidence was obtained to demonstrate the presence of a more vigorous acute systemic inflammatory reaction to cadmium administration in male rats compared to female rats.

MATERIALS AND METHODS

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. Six to eight animals were assigned to each treatment group in each of at least three independent experiments. Cadmium chloride (Serva, Feinbiochemica, Germany) prepared in sterile pyrogen-free saline was administered *i.p.* in a dosing volume of 0.5 mL and in concentration calculated so that the animals received 0.5, 1, or 2 mg of cadmium/kg body weight (b.w.). Pyrogen-free saline alone was administered to control animals. All measurements were carried out 24 hours following cadmium administration, in animals anesthetized with sodium thiopentone (40 mg/kg/b.w. *i.p.*).

Clinical Biochemistry

Serum aspartate amino transferase (AST) activity was determined using an autoanalyzer (Ciba Corning Express, Oberline, USA) and commercially available reagents. Urine (24-hour) was collected from rats placed in individual metabolic cages immediately after cadmium administration. Concentrations of creatinine in the serum and daily urine were determined with an automatic multichannel analyzer (Beckman-Astra, Irvine, USA) using commercially supplied reagents.

Soluble Indicators of Inflammation (TNF, IL-6, and α_2M)

Immunoreactive plasma TNF and IL-6 were measured by solid phase enzyme-linked immunosorbent assay (ELISA; Bender Med Systems, Austria for rat TNF and R&D Systems, USA for rat IL-6). Bioactivity of IL-6 in plasma was determined by the proliferation (evaluated by MTT reduction assay) of an IL-6-dependent murine hybridoma cell line as described[24]. Plasma concentrations of α_2M were measured by rocket immunoelectrophoresis, according to Baumann[25], with the use of polyclonal rabbit anti-human α_2M antibody (Dako, USA) cross-reactive to the rat protein. The relative concentrations of alpha$_2$-M were established by quantification of the areas under the respective immunoprecipitation peaks. Results are expressed as the relative increase, calculated as percentage of the value obtained in control (cadmium 0 mg/kg b.w.) animals, which were considered as 100%.

Cellular Indicators of Inflammation

Total leukocyte counts were determined with an improved Neubauer hemocytometer. Differential leukocyte counts were determined by differentiating at least 200 cells from air-dried whole blood smears stained according to the May Grünwald-Giemsa (MGG) protocol.

State of leukocyte aggregability was estimated by a direct slide test[26], by recording aggregated leukocytes in fine films. Cells were considered aggregated when three or more nuclei were placed less than one cell diameter apart.

Peripheral blood granulocyte assays were performed in cells isolated from the heparinized blood by dextrane sedimentation and centrifugation on NycoPrep animal 1.077 (Nycomed AS, Norway) density gradient. Granulocyte priming was evaluated by cytochemical assay for the respiratory burst[27] and

adhesion of granulocytes to non-cellular matrix[28], based upon their phorbol-12-myristate-13-acetate (PMA)-stimulated ability to reduce the tetrazolium salt ntiroblue tetrazolium (NBT; Sigma-Aldrich, USA) or to adhere to plastic. Cell culture medium RPMI 1640 (ICN Pharmaceuticals, USA) supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS) was used in cell culture experiments. In experiments where the neutrophil-priming capacity of plasma was tested, cells were incubated with 10% plasma samples instead of FCS.

Data Display and Statistical Analysis

Results are expressed as $\bar{x} \pm s$ for *ex vivo* measurements or as mean ± SE for cell culture experiments. Statistical significance was defined by Mann-Whitney U test (for *ex vivo* evaluations) or by Student's *t*-test (for *in vitro* cell culture experiments). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Administration of high (2 mg/kg b.w.) and moderate (1 mg/kg b.w.) doses of cadmium resulted in significantly increased values of AST in male rats (199±47 U/mL and 113±4 U/mL, respectively *vs* 77±27 U/mL in controls) and increased creatinine clearance (53±3 mL/h/100 g b.w. and 49±5 mL/h/100 g b.w., respectively *vs* 38±4 mL/h/100 g b.w. in controls), compared to no change in female rats. No effect was seen either in male rats or in female rats when a low cadmium dose (0.5 mg/kg b.w.) was administered. The mortality rate was 33.3% in male rats and 28.6% in female rats at a high Cd dose. Consequently, a dose of 1 mg of cadmium/kg bw was chosen for the investigation of sex differences in cadmium-evoked inflammatory response.

Determination of TNF and IL-6 in general circulation of rats revealed no measurable immunoreactive TNF or IL-6 in the plasma of control animals of either sex. Administration of cadmium resulted in an increase in the levels of these cytokines (Fig. 1A). While the increase in TNF was similar in animals of both sexes. The circulating IL-6 concentration was significantly higher noted in male rats than female rats (P<0.02). Cadmium administration resulted in a tremendous increase of IL-6 bioactivity in the plasma of cadmium-treated male rats, compared to that in female rats (Fig. 1B). α_2M was increased 409%±93% in male rats following cadmium administration and 114%±34%) in female rats (Fig. 1C).

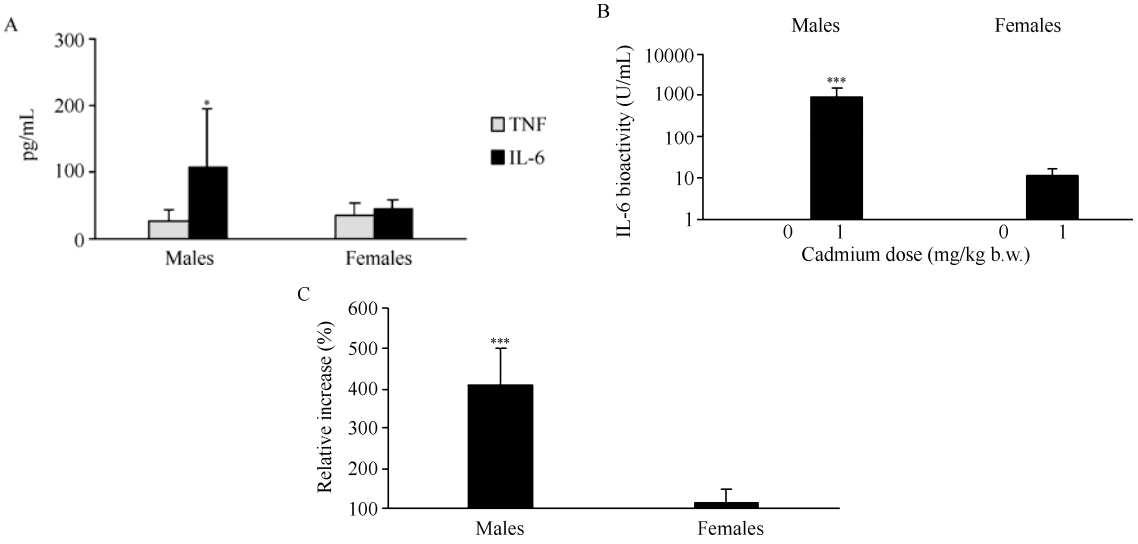

FIG. 1. Plasma levels of soluble indicators of inflammation in male and female rats following cadmium administration. (A) Immunoreactive TNF and IL-6 levels; (B) Bioactive IL-6 levels; (C) Relative concentrations of alpha-2 macroglobulin. Results are expressed as $\bar{x} \pm s$ from six to ten animals. $^{*}P$<0.02, and $^{***}P$<0.001 *vs* control animals.

Intraperitoneal administration of cadmium chloride in male rats led to leukocytosis, accounting for the increased number of granulocytes (Table 1). A small but significant rise in the relative number of neutrophils and a slight but statistically significant decrease in the relative number of lymphocytes in female rats did change the total peripheral blood leukocyte count following cadmium administration. An increase in the relative number of immature neutrophils was noted in male rats that received cadmium, compared to control male rats. A significant increase in these forms of neutrophils, but

of lesser magnitude than that in male rats, was noted in female rats that received cadmium. Evaluation of the state of leukocyte aggregability *in vivo* revealed the increased number of peripheral blood cells found in aggregates in male rats after cadmum administration, compared to a negligible increase in female rats (Fig. 3). The aggregates were composed mainly of neutrophils (Fig. 3).

TABLE 1

Quantitative Changes in Peripheral Blood Leukocytes ($\bar{x} \pm s$)

Cadmium Dose (mg/kg b.w.)	Male Rats		Female Rats	
	0	1	0	1
Total Leukocytes ($\times 10^9$/l)	7.1±3.0	19.9±12.2**	6.6±3.0	6.4±3.0
Neutrophils (%)	14.7±9.5	62.9±30.4***	20.2±5.5	29.2±4.6***
Lymphocytes (%)	82.8±9.3	35.2±30.0***	76.3±4.6	66.6±3.4***
Immature Neutrophils (%)	0.41±0.40	4.31±2.91***	0.69±0.31	1.29±0.80*

Note. *P<0.05, **P<0.01, ***P<0.001 *vs* same-sex controls (cadmium 0 mg/kg b.w.).

Analysis of peripheral blood PMN priming in male and female rats following cadmium administration demonstrated an increased PMA-induced NBT reduction in granulocyte cultures from cadmium-treated animals, to levels significantly different from those of controls in both male and female rats. However, much higher levels were reached in the cultures of cells from male rats (Fig. 2A). PMA-stimulated adhesion was significantly higher in cells from male rats treated with cadmium than in controls, with no differences seen in granulocyte cultures from female rats (Fig. 2B).

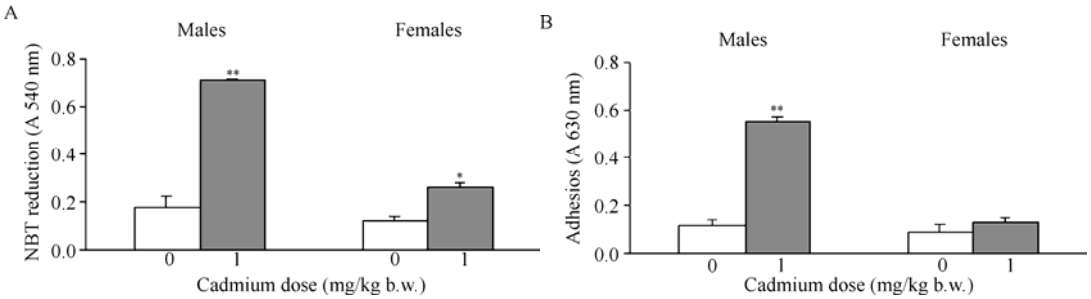

FIG. 2. Peripheral blood neutrophil priming in male and female rats following cadmium administration. (A) NBT reduction expressed as absorbance at 540 nm; (B) Adhesion expressed as absorbance at 630 nm. Each bar represents the $\bar{x} \pm s$ of quadruplicate cell cultures from a single experiment. *P<0.05 and **P<0.01 *vs* cells from control animals.

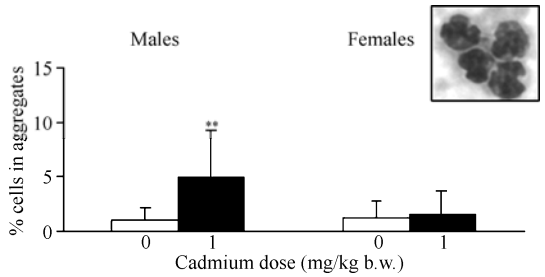

FIG. 3. Relative number of aggregated leukocytes and granulocytes in aggregates (MGG, 20×). Results are expressed as $\bar{x} \pm s$ from eight to ten animals. **P<0.01 *vs* control animals.

When the effect of plasma from cadmium-treated male and female rats on naive PMN priming was assessed (Table 2), a higher potency of plasma from male rats to which cadmium was administered to enhance the PMA-stimulated NBT reduction in naive male or female PMN was noted, compared to the activation of the same-sex PMN in the presence of same-sex plasma from controls. A slight increase in NBT reduction (P=0.05) was observed when naive male PMN was cultured in the presence of plasma from Cd-treated female rats. No increase in NBT reduction by naive female PMN was noted when the plasma of Cd-treated female rats was used.

TABLE 2

Priming Effect of Plasma on Peripheral Blood Granulocyte PMA-induced Activation in Male and Female Rat ($\bar{x} \pm s$)

Plasma Source	Male PMN	Female PMN
Male, Control	0.048±0.010	0.056±0.002
Male, Cd-treated	0.100±0.001**	0.096±0.007**
Female, Control	0.052±0.005	0.053±0.007
Female, Cd-treated	0.069±0.012[a]	0.059±0.002

Note. **P<0.01 and [a] P=0.05 *vs* NBT reduction by same-sex PMN in the presence of same-sex control plasma.

DISCUSSION

In this study, sex differences in systemic inflammatory response to intraperitoneal cadmium administration were evaluated by comparing selected soluble and cellular parameters of inflammation in rats.

Increase in circulating levels of TNF and IL-6 is consistent with our previous study, showing an increase in cytokine bioactivity in male rats following cadmum administration[21-22]. As hepatic and renal tissue and cell IL-6 production have been shown in cadmium-treated mice[8-10] and peripheral blood cells[29], a greater production by these sources might have contributed to more highly increased plasma levels of this cytokine in male rats compared to those in female rats. Sex differences in IL-6 production were detected by bioassay as well. Tremendous rise of IL-6 bioactivity in the plasma of male rats might be due to the presence of a greater quantity of soluble IL-6 receptor, since B9 bioassay for IL-6 detects its presence[30]. Given the data suggesting that the activity of IL-6 is greatly enhanced by soluble forms of its receptors[31], cadmium-induced increase of IL-6 activity might have broader biological implications for the toxicity of this metal. Gender differences in systemic IL-6 response shown in this study are consistent with the data demonstrating a greater increase in circulating IL-6 levels in male rats in the settings of inflammation induced by tissue injury[32] or by microbial products[33]. Gonadal hormones may be a relevant underlying mechanism, as estrogen can suppress systemic interleukin-6 production in mice in the settings of inflammation[33-34]. A similar production of TNF by target tissues and cells of cadmium-treated rats might be due to the absence of gender differences in plasma levels of this cytokine. Huge increase of the α_2M levels in the circulation of male rats is in agreement with the greater inducibility of acute phase proteins in male rats compared to female rats, as shown in systemic inflammation evoked by turpentine administration[35]. The observed increase might have resulted from cadmium-stimulated α_2M production by the liver, as demonstrated in other acute phase proteins of cadmium-treated mice[8] and rats[36].

Increased production/release of neutrophils from bone marrow might account for their rise in animals of both sexes. The increased number of immature neutrophils (present study) and accelerated regeneration of granulocyte-macrophage progenitor cells in irradiated mice that received cadmium[37] support such an assumption. Increase in TNF and IL-6, known hematopoiesis-relevant cytokines[38-39], might be involved in the shift of peripheral blood cell types in cadmium-treated animals. Both IL-6-induced bone marrow neutrophil release[39] and mobilization of neutrophils from the marginal into the circulating pool[40] might have contributed to the observed high *vs* moderate rise of neutrophils in male rats *vs* female rats. The state of leukocyte aggregability *in vivo*, a phenomenon known as leukergy[26], is a non-specific indicator of inflammation used as a method to screen the presence of systemic inflammation and measure its intensity[41]. Therefore, the increased relative number of peripheral blood leukocytes found in aggregates of cadmium-treated male rats demonstrate the presence of more intense systemic inflammation than that found in female rats.

A higher responsiveness to exogenous (PMA) stimulation, i.e. neutrophil priming, in Cd-treated male rats, is in agreement with the data that demonstrate a higher peripheral blood neutrophil activity of systemic inflammation evoked by traumatic tissue injury in male rats[42-43]. The significant priming effect of plasma from cadmium-treated male rats *vs* low or absent effect of plasma from cadmium-treated female rats on naive peripheral blood neutrophils suggests the role of blood-borne inflammatory mediators in gender differences in neutrophil function. Both IL-6 and TNF involved in various aspects of neutrophil response, along with other inflammagens in the circulation, might be responsible for the observed difference. Signaling pathways involved in neutrophil function might contribute to differential neutrophil response as well, as shown for higher receptor-triggered effector neutrophil activity in male rats in the settings of trauma-induced systemic inflammation[42]. Contribution of a direct effect of cadmium could, however, not be excluded, since a greater adhesion capacity to plastic has been shown in human peripheral blood neutrophils in the presence of exogenous cadmium[44]. Gender differences in neutrophil response may rest on gonadal hormones, including estrogen suppressing neutrophil functions in mice[33] and humans[45].

In conclusion, cadmium administration induces systemic inflammation that is more intense in male rats then in female rats. The biological consequences of such a differential response may include a less vulnerability of female rats to cadmium-induced inflammation-mediated tissue injury and a greater vulnerability to concurrent infections than those of male rats. These possibilities warrant future investigation.

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