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TOLUENE MEDIATED OXIDATIVE STRESS AND GRANULO-MONOCYTOPOIESIS

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The influence of toluene exposure on some biochemical and hematological parameters was investigated in adult female Wistar rats. The animals were subjected to intraperitoneal administration of toluene diluted in propylene glycol and the diluent alone for 3, 7 and 11 consecutive days at the same time intervals. The effects of toluene and propylene glycol were evaluated biochemically by determining the plasma concentrations of total proteins, albumin and ceruloplasmin (Cp) together with erythrocyte malondialdehyde (MDA), and hematological indices from peripheral blood and bone marrow. The biochemical acute phase response was manifested by an elevated Cp concentration in all experimental animals. The markedly enhanced MDA concentration and statistically significant decrease in albumin level in toluene treated rats, indicated damage, to blood vessel endothelia. Alterations of leukocytes of peripheral blood and bone marrow (BM) granulocytic-monocytic progenitor cells were typical of an inflammatory response, with stimulation of granulo-monocytopoiesis. Therefore, it can be assumed that both toluene and propylene glycol mediated sterile peritonitis and oxidative stress injury, with changes intensified by toluene action.

Key words: rat, ceruloplasmin, hematological indices, malondialdehyde, propylene glycol, toluene

INTRODUCTION

Individuals are exposed to oxidative stress even before birth. Sources of oxidative stress are ubiquitous and include, in particular, the oxidative burst of neutrophils, ischemia-reperfusion events, cytochrome P450 action, prostaglandins, xenobiotics, aerobic or long duration exercise, emotional stress etc. (Chen *et al*., 2003; Cichetti and Argentin, 2003; Ajmani *et al.*, 2003; Tauler *et al.*, 2002). Oxidative stress can also ensue from environmental causes, such as UV radiation, pollution (Grella *et al.*, 2002), cigarette smoke (Kim *et al*., 2003), as well as from some medical interventions, like laparoscopy and open surgery (Bentes De Souza *et al.*, 2003).

Toluene is a ubiquitous chemical commonly used for its solvent properties in industry and manufacturing. For that reason there is a potential for both occupational and nonoccupational exposure to it. The influence of toluene has been examined in many studies, with contradictory results and discrepancies concerning the interpretation of adverse effects (Wang *et al*., 1996; Nedelcheva, 1996; Neghab and Stacey, 1997; Pedersen and Rasmussen, 1982). It is known that toluene and its metabolites, benzyl alcohol and hippuric acid (Smith-Kielland *et al.*, 1993; Backes *et al*., 1993; Paguotto *et al.*, 1967), are capable of inducing oxidative stress generating reactive oxygen (ROS) and nitrogen species (RNS) in the liver, central nervous system, lung and kidney, with consequent tissue damage (Hauffman *et al.*, 1997; Tamizhselvi *et al.*, 1995; Mattia *et al.*, 1993a; Mattia *et al.*, 1993b; Myhre *et al.*, 2001).

We focused our investigation on the acute effects of toluene *in vivo*, with respect to its ROS enhancing potential, and influence on some plasma proteins and cells in bone marrow and blood, including its impact on granulocyte-monocyte progenitors (CFU-GM).

MATERIAL AND METHODS

Animals

Adult female Wistar rats, weighing between 250-300g, 2.5-3 months old (Institute for Medical Research, Belgrade) were kept in cages with free access to food pellets and water. All experiments were performed in accordance to the principles and guidelines of the Canadian Council on Animal Care /CCAC/. Rats were anaesthetized by intramuscular application of 0.5 ml of a mixture of ketamine, ketalar and formidal in doses of 75 mg/kg b.m., 15mg/kg b.m. and 0.75 mg/kg b.m., respectively.

Experimental design

The animals were divided into three groups: a negative control group of eight untreated rats (*group C*); a control group of rats, which received propylene glycol (0.3 ml/200 g b.m.; n=24) (*group PG*) and *group T* containing rats which received toluene (0.7 μm) diluted in propylene glycol (0.3 ml/200 g b.m.; n=24). Groups PG and T were treated intraperitoneally daily for 3, 7 or 11 days, and were sacrificed on the respective following day at definite time intervals.

Blood was obtained directly by heart puncture. EDTE plasma was separated from whole blood after centrifugation at 3000 rpm, and immediately stored at -20^{o}C until the tests were done.

Biochemical measurements

The oxidase activity of Cp in plasma was obtained using a colorimetric enzyme assay (Sunderman *et al*., 1970) using a spectrophotometer (Spekord M40, Karl Zeiss, Jena) at 530 nm.

Total protein and albumin concentrations were estimated by the colorimetric albumin-BCG (brom-cresol-green) method, at 628 nm (25^{o}C, pH 4.2; Basic Spec-

trophotometric Biochemical Analyser, Secomam, France). Erythrocyte MDA concentrations were determined using a colorimetric procedure (Uchiyama *et al.*, 1978) at 535 nm on a spectrophotometer (Spekord M40, Karl Zeiss, Jena). Haemoglobin concentration was detected colorimetrically (Tentori *et al.*, 1981), at 540 nm on a spectrophotometer (Spekord M40, Karl Zeiss, Jena).

Determination of peripheral blood parameters

Total leukocytes were enumerated manually using a haemocytometer after suspension in Turk solution. Blood smears were stained with May-Grunwald-Giemsa, and differential cell counts were made (at least 100 cells/counted sample).

Determination of granulocyte-macrophage progenitor cells – CFU-GM

Bone marrow cells were flushed out of the femurs and suspended in Dulbecco's Modification of Eagle's Medium (DMEM, GibcoBRL, Life Technologies, Paisley, Scotland). Bone marrow nucleated cells were counted using the same procedure applied for total peripheral blood leukocytes.

The viability of cells was determined using the trypan-blue exclusion test.

Colony forming assay for granulocyte-macrophage progenitor cells – CFU-GM

Clonal assays were performed essentially as previously described (Stojanović *et al.*, 1988). Cells recovered directly from donor animals were plated in 35 mm Petri dishes in a culture medium containing 0.9% methylcellulose, 20% of fetal calf serum (Methocult GF M 3434 Stem Cell Technologies, Vancouver, Canada) and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air (Haereus incubator, type I). CFU-GM derived colonies were counted on day 7, under an inverted microscope (magnification x 50) as groups with more than 50 cells.

Statistical analyses

The data are expressed as arithmetic mean ± standard error. One way analysis of variance (ANOVA) was applied. When the results of ANOVA were significant, Student's t-test (two-sample assuming equal variance) was performed to determine the level of significance and probability.

RESULTS

Biochemical parameters

In the course of the experiment, total protein concentration remained in the physiological range (data not shown), while albumin levels were significantly depressed ($p<0.001$) in plasma from all treated groups compared to the control animals (Fig 1).

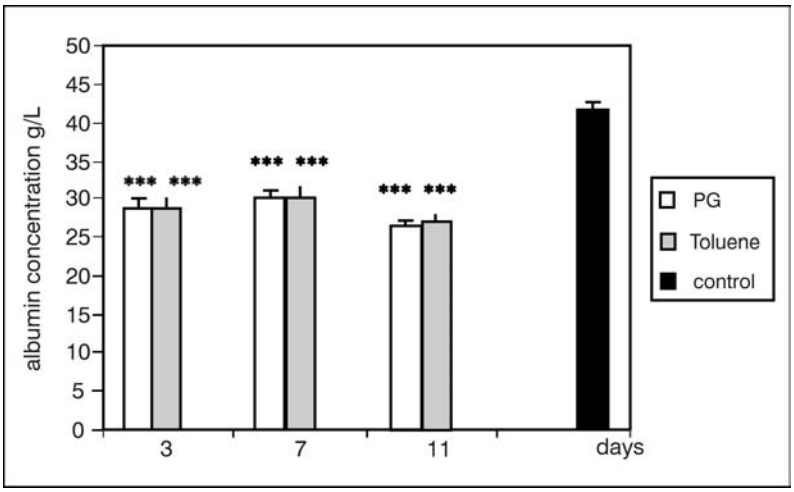


Figure 1. Effect of toluene application on plasma albumin concentration in rats.
Values are presented as mean ± SE for 8 animals.
(***) *p<0.001* vs control

Mean ceruloplasmin concentration showed a statistically significant increase after 7 days of application in group PG ($p<0.001$) and group T ($p<0.05$), compared to that in the untreated rats (Figure 2). Changes in erythrocyte MDA were detected only after toluene treatment, reaching a 5-fold increase after 11 days of treatment, in comparison to the control and PG treated rats (Figure 3).

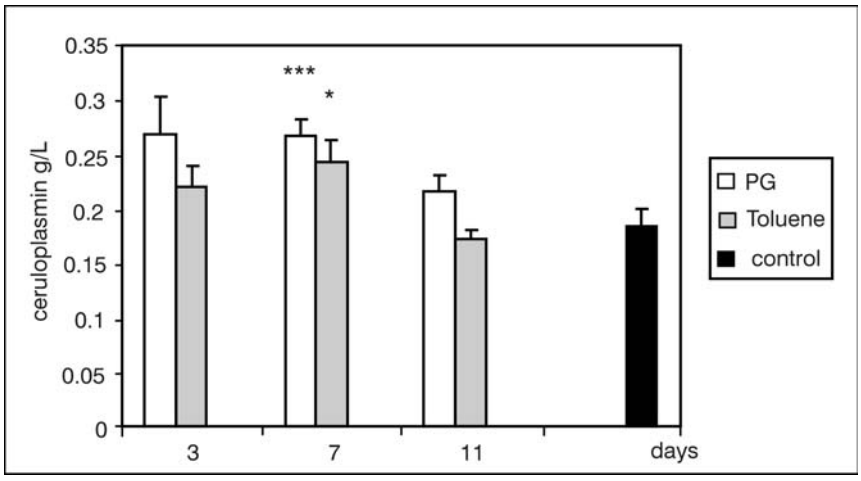


Figure 2. Effect of toluene application on plasma Cp concentration in rats.
Values are presented as mean ± SE for 8 animals.
(***) *p<0.001* and (*) *p<0.05* vs control

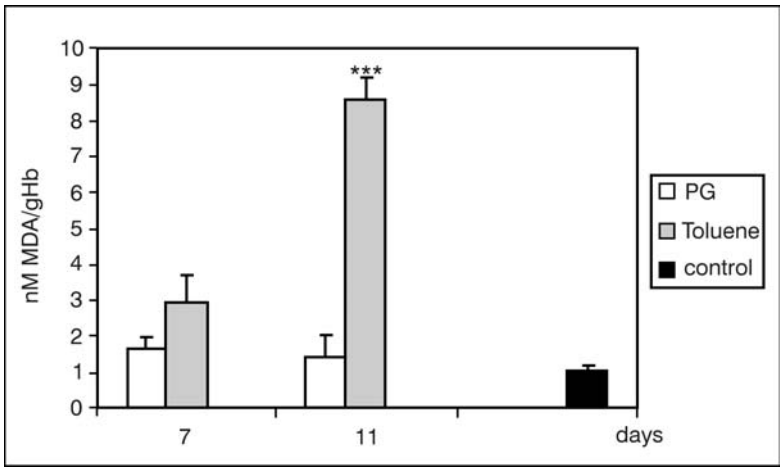


Figure 3. Effect of toluene application on erythrocyte MDA concentration in rats.
Values are presented as mean ± SE for 8 animals.
(***) $p<0.001$ vs control

Blood and marrow cellular reaction.

The peripheral blood parameters in the control group were in the physiological range for this species (data not shown).

Table 1. Effect of toluene application on rat white blood cells number.

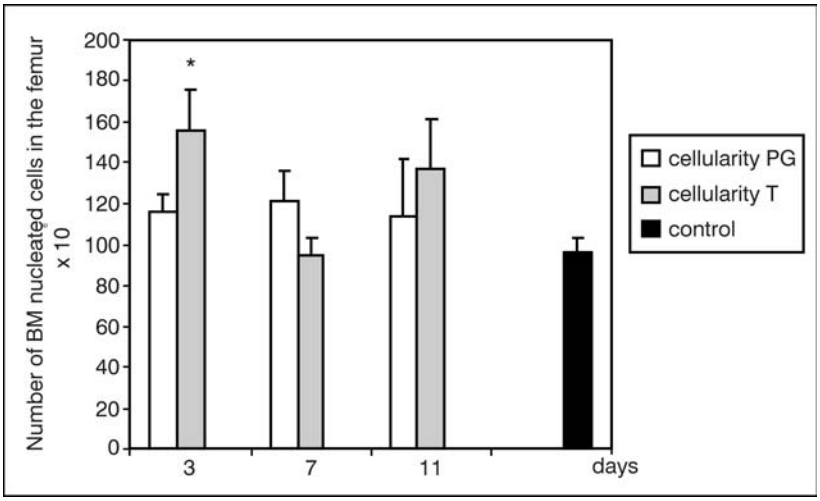
Day of application	3rd		7th		11th		Control
	Toluene	PG	Toluene	PG	Toluene	PG	
Total leukocytes (x 10^9/L)	15.8±1.8	8.6±0.9***	23.3±3.0	18.0±2.0	19.9±2.3	17.7±1.9	16.9±1.1
Lymphocytes (x 10^9/L)	11.6±1.4	6.3±0.6***	15.5±2.1	12.6±1.4	13.8±1.9	13.0±0.8	14.4±1.1
Neutrophils (x 10^9/L)	3.2±0.5**	1.6±0.2	5.7±1.2**	4.0±0.8**	4.6±0.9**	3.1±0.6*	1.6±0.1
Monocytes (x 10^9/L)	0.8±0.1	0.5±0.2	2.1±0.3***	1.3±0.1**	1.4±0.3	1.2±0.5	0.8±0.09

Values are presented as mean ± SE for 8 animals.
(***) $p<0.001$, (**) $p<0.01$ and (*) $p<0.05$ vs control.

PG treatment induced a transient leukopaenia (Table 1) after 3 days, $p<0.001$ (mostly by a decrease in lymphocyte number), without visible changes

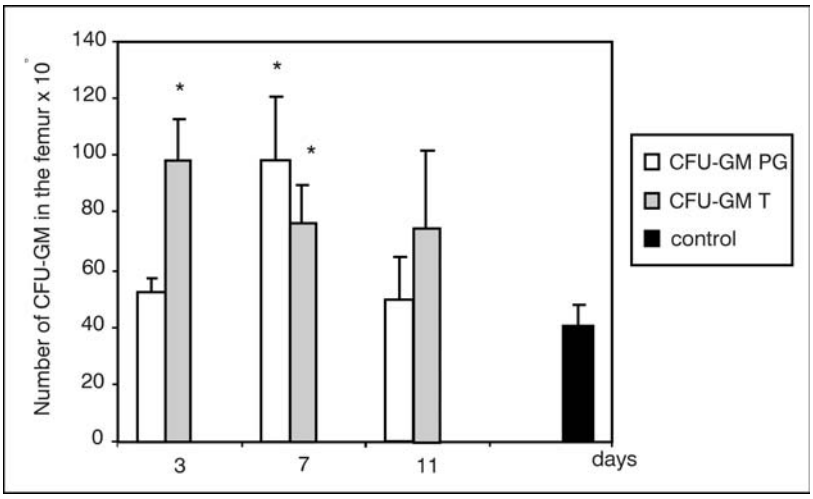
of total BM cells (Figure 4A) and CFU-GM populations (Fig 4B). This onset of leukopoenia was compensated after 7 days treatment (Table 1), but with an increase in the relative proportion of peripheral blood neutrophils (4.049±0.750, n=8) and monocytes (1.309±0.121; n=8). This compensation seems to be the result of a

A



Values are presented as mean ± SE for 8 animals.
(*) $p<0.05$ vs control

B



Values are presented as mean ± SE of 8 animals.
(*) $p<0.05$ vs control

Figure 4. Effect of toluene application on rat bone marrow cellularity (A) and on the number of CFU-GM (B).

progenitor cell compartment activation, since an increased number of CFU-GM per femur was detected after 7 days treatment (Fig 4B), although the increase in total number of bone marrow cells did not reach a statistically significant level (Fig 4A).

In contrast, toluene prevented this leukopenic effect of PG treatment (Table1). Furthermore, it provoked a rapid neutrophil (since day 3) and monocyte (day 7) increase, without affecting the total lymphocyte number (Table 1). These effects, observed in peripheral blood, were probably due to an effective stimulation of bone marrow GM progenitors, that probably occured early (at day 3 CFU-GM number doubled - Fig 4B) and the total number of BM cells increased significantly (Fig 4A). Furthermore, the increased number of BM CFU-GM persisted for at least 7 days (Fig 4B).

DISCUSSION

Malondialdehyde (MDA) is one of the end-products of the peroxidation of membrane lipids caused by ROS formation (Patockova et al., 2003), especially by the superoxide ion. It is currently considered to be a basic marker of oxidative stress. The marked rise in red blood cell MDA concentration after toluene treatment observed here indicates uncured cellular damage induced by ROS induction, as previously seen by Myhre *et al.*, (2001), Mattia *et al.*, (1993a) and Mattia *et al.* (1993b). The decline in albumin concentration could be ascribed to increased endothelial barrier permeability (Tamizhselvi *et al.*, 1995). However, this phenomenon was also detected in the absence of toluene treatment and under the condition of low erythrocyte MDA levels. Therefore, possible vessel damage was not provoked solely by the influence of O_2 radicals and it could rather be the result of an inflammatory reaction. Moszczynski and Coworkers (1983a; 1983b; 1980) showed reduction of intact lymphocyte lysosomes and intensified metabolic activity of peripheral blood neutrophils, with diminished peroxidase activity.

Accelerated synthesis of ceruloplasmin with a significant rise in mean concentration in group T rats is associated with the liver and possibly to activated monocytes of peripheral blood (Mazumder *et al.*, 1997; Gaitskhoki *et al.*, 1990). As the increased Cp level was accompanied by a low erythrocyte MDA concentration, we may infer a possible protective role against oxidizing agents (Reyes and Holmgren, 1991; Yang *et al.*, 1993; Saenko *et al.*, 1990). Further investigations should be made to elucidate this question.

At the very start of the acute phase response the body has an increased need for mature, functional cells of granulocyte-monocyte origin, at first satisfied by mobilizing mature cells from the marginal pools and later from the reserve compartment of the bone marrow. This is rapidly compensated by de novo production. This typical response of the granulo-monocytic lineage, best analyzed by a rat model of sterile peritonitis (Jovčić *et al.*, 1993; Milenković *et al.*, 1993), was evident in the toluene-treated animals. Since this *in vivo* cellular response occurs in the context of an inflammatory reaction, the cellular changes (stimulation of the progenitor compartment) may be explained by reactive induction of stimulatory factors and cytokine production (Stojanović *et al.*, 1988; Milenković, 1993). This

could be amplified by oxidative stress, which also induces the production of tumour necrosis factor (TNF), interleukin (IL)-1 and IL-6 (Vassilakopoulos *et al.*, 2003). As MDA increases, the oxidative stress most probably occurs after toluene administration. This could be the major (albeit not exclusive) explanation for our results showing acute stimulation of medullar granulo-monocytopoiesis by toluene treatment.

In conclusion, our results confirm that toluene induces oxidative stress. Furthermore, the changes induced by ROS formation extend to bone marrow progenitors, a phenomenon never described before. Although the concentrations of pro-inflammatory cytokines especially IL-1, IL-3, Il-6 and TNF were not determined in this work, their induction by toluene treatment is very probable, which might explain the stimulation of BM CFU-GM found here.

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TOLUENOM POSREDOVANI OKSIDATIVNI STRES I GRANULO-MONOCITOPOEZA

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SADRŽAJ

U ovom ogledu je ispitivan efekat toluena na biohemijske i hematološke parametre odraslih ženki Wistar pacova. Životinjama je intraperitonealno davan rastvor toluena u propilen glikolu, kao i propilen glikol, jednom dnevno u trajanju od 3, 7 i 11 dana. Efekti davanih rastvora odredjivani su biohemijskim i hematološkim ispitivanjima plazme, krvi i punktata kostne srži. U plazmi pacova određivani su koncentracije ukupnih proteina, albumina kao i koncentracija eritrocitnog malondialdehida (RBC MDA). Biohemijske promene manifestovale su se hipoalbuminemijom, porastom koncentracije ceruloplazmina i visokom koncentracijom RBC MDA. Ovi nalazi ukazuju na oštećenje endotela krvnih sudova praćeno odgovorom akutne faze. Promene u broju leukocita periferne krvi kao i povećanje broja granulocitno-monocitnih (CFU-GM) progenitora kostne srži takođe su ukazali na tipičan proinflamatorni odgovor.

Naši rezultati potvrđuju da toluen izaziva oksidativni stres i sterilni akutni peritonitis. Promene izazvane slobodnim radikalima kiseonika utiču i na odgovor progenitora kostne srži, pojavu koja do sada nije bila opisana.