

**EFFECTS OF INTRAPERITONEAL APPLICATION OF TOLUENE DISSOLVED IN PROPYLENE GLYCOL ON ERYTHROPOIESIS IN WISTAR RATS**

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*The potential toxic effect of toluene on erythropoiesis was investigated during the enhanced erythropoietic activity provoked by application of propylene glycol (known as a mild hemolytic agent) in adult female Wistar rats. The animals were treated daily for 3, 7 or 11 days, with an intraperitoneal dose of toluene dissolved in propylene glycol (T+PG) or propylene glycol (PG) alone. The effects of T+PG and PG on some hematological parameters in peripheral blood and bone marrow were evaluated at the time of sacrifice.*

*The number of red blood cells (RBC), reticulocytes (Rt), hematocrit values (PCV), and hemoglobin concentration (Hb) were determined in peripheral blood samples using standard laboratory procedures. Total bone marrow nucleated cells (TBMNC) were counted and the myelogram was analysed as well. The number of early bone marrow erythroid progenitor cells - Burst Forming Unit - Erythroid (BFU-E) was assessed using a colony forming assay on methyl-cellulose.*

*Toluene dissolved in PG induced a decrease in RBC number, PCV and Hb concentration and an increase within the bone marrow BFU-E and precursor erythroid cell compartments, as well as the percentage of peripheral blood Rt, indicating enhanced erythropoietic activity. Very similar changes occurred when PG was administered alone. Therefore, it can be assumed that short-term application of a low dose of toluene (3 mg/kg) dissolved in PG did not have a toxic effect during PG induced enhanced erythropoietic activity.*

*Key words: BFU-E, hematological parameters, myelogram, propylene glycol, rat, toluene.*

**INTRODUCTION**

Toluene (T) is an aromatic hydrocarbon, commonly used in industry due to its solvent properties. Examination of the influence of toluene on health has been the subject of many studies, but with contradictory results and discrepancies concerning the interpretation of adverse effects (Pedersen and Rasmussen, 1982; Wang *et al.*, 1996; Nedelcheva, 1996; Neghab and Stacey, 1997). It is known that

toluene, as well as its metabolites, benzyl alcohol and hippuric acid, can induce oxidative stress and generate reactive oxygen (ROS) and nitrogen species (RNS) in the liver, CNS, lung, and kidney (Paguotto *et al.*, 1967; Backes *et al.*, 1993; Smith-Kielland *et al.*, 1993), followed by consequent tissue damage (Mattia *et al.*, 1993a; 1993b, Tamizhselvi *et al.*, 1995; Hauffman *et al.*, 1997; Myhre *et al.*, 2001). However, data concerning the hematological toxicity of toluene are contradictory. Some investigators (Hsieh *et al.*, 1989; Bell, 1989) reported that toluene had no potent hematotoxic effects, but isolated reports demonstrated bone marrow damage (Cuneo *et al.*, 1999) and anemia after toluene exposure (Garavini and Seren, 1979). It was also found that toluene increased ROS production, induced hemoglobin modification and changes in erythrocyte metabolism (Borozan *et al.*, 1995).

Propylene glycol (PG) is a widely used organic solvent for parenteral drugs. We used it, as a solvent to deliver hydrophobic toluene into the rat intraperitoneal cavity. PG mediated hemolytic anemia is well documented in cats fed with PG containing diets (Christopher *et al.*, 1989), as well as in humans and laboratory animals receiving parenteral PG (Mottu *et al.*, 2001).

In the present experiment PG was used to stimulate the erythroid lineage. We used this model of enhanced erythropoiesis, to evaluate the potential acute toxic effect of toluene on erythropoiesis. We investigated changes within the erythroid progenitor and precursor cell compartments in the bone marrow as well as changes in red blood cell (RBC) counts in peripheral blood, after 3, 7 and 11 days of PG and T+PG application.

## MATERIAL AND METHODS

### *Animals*

Adult female Wistar rats, weighing between 250-300g and 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 with the principles and guidelines of the Canadian Council on Animal Care /CCAC/. Rats were anaesthetized by an intramuscular application of 0.5 ml of a mixture of ketamine, ketalar, and formidal, in doses of 75 mg/kg b.m., 15 mg/kg b.m. and 0.75 mg/kg b.m., respectively.

### *Experimental design*

Three groups of animals were observed: a negative control group of eight untreated rats, *C group*; a second control group of rats, which received propylene glycol (n=24) – *PG group* and a group of rats which received toluene dissolved in propylene glycol (n=24) – *T+PG group*. PG (1.56 g/kg b.m.) was given intraperitoneally at 0.3 ml/200 g b.m, while T+PG rats received the same dose containing toluene (3 mg/kg b.m.). The groups were treated daily for 3, 7 and 11 days, and were sacrificed in definite time intervals, respectively.

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

#### *Determination of peripheral blood and bone marrow parameters*

The number of red blood cells (RBC), reticulocytes (Rt), hematocrit values (PCV), and haemoglobin concentration (Hb) were determined in peripheral blood samples using standard laboratory procedures. Hb concentration was measured using a spectrophotometric assay (Tentori *et al.*, 1981), at 540 nm (Spekord M40, Karl Zeiss, Jena). RBC were enumerated manually using a haemocytometer after suspension in Hayem solution. PCV was determined by the microhematocrit method. The number of reticulocytes was estimated per 1000 RBC on peripheral blood smears stained with brilliant cresyl blue. Rt index was calculated from the following formula:  $Rt\ index = PCV \times Rt\ (\%) / control\ PCV$ .

Bone marrow cells were flushed out of the femurs and then suspended in Dulbecco's Modification of Eagle's Medium (DMEM, GibcoBRL, Life Technologies, Paisley, Scotland). Total bone marrow nucleated cells (TBMNC) were counted manually, using a hemocytometer after suspension in Turk solution. The viability of cells was determined using the trypan-blue exclusion test. On bone marrow smears stained by the May-Grunwald-Giemsa procedure, 1000 nucleated cells were differentiated and divided into compartments comprising proliferative erythroblasts (ERBL) and non proliferative orthochromatic blasts (ORTHO). The myeloid/erythroid ratio (M:E) was calculated from the percentages of myeloid (sum of granulocytes, metamyelocytes, myelocytes and monocytes) and erythroid (ERBL + ORTHO) cells.

#### *Colony forming assay for erythrocyte progenitor cells – BFU-E (Burst forming unit-erythroid)*

Clonal assays were performed essentially as previously described (Petakov *et al.*, 1998). Bone marrow cells from the experimental rats 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<sub>2</sub> in air – (Haereus incubator). BFU-E derived colonies were counted on day 7, under an inverted microscope (magnification x 50) as groups with more than 100 cells.

#### *Statistical analyses*

The data are expressed as arithmetic mean ± standard error. Student's t-test: two-samples assuming equal variance, was performed to determine level of significance.

## RESULTS

To elucidate the potential toxic effect of toluene on erythropoiesis, erythroid cells were examined at various stages of differentiation and maturation and at different time points during toluene application.

#### *Bone marrow*

To obtain data concerning the rearrangement of erythroid progenitor cells and precursor cell compartments of the bone marrow we determined the TBMNC per femur, myelogram and number of BFU-E (Table 1, Fig. 1 and Fig. 2).

Table 1. The effect of application of PG and T+PG for 3, 7 and 11 days on rat femur TBMNC and myeloid and erythroid cell compartment ratio (M:E)

Days of application	3		7		11		
	Control	PG	T+PG	PG	T+PG	PG	T+PG
n = 8	960 ± 51	1250 ± 52 <sup>c</sup>	1547 ± 133 <sup>cx</sup>	1210 ± 96 <sup>b</sup>	960 ± 48 <sup>ay</sup>	1133 ± 19 <sup>a</sup>	1350 ± 22 <sup>bx</sup>
TMNC x 10 <sup>5</sup>	2:1	2.3:1	2:1	2.9:1	2:1	2.3:1	1.6:1

Values are presented as mean ± SE.

Significance: PG and T+PG vs. control: (a) not significant, (b)  $p < 0.05$ , (c)  $p < 0.01$ , (d)  $p < 0.001$

Significance: PG+T vs. PG: (x) not significant, (y)  $p < 0.05$

Table 2. The complete blood counts of rats treated daily with PG or T+PG for 3, 7 and 11 days, respectively

Days of application	3		7		11		
	Control	PG	T+PG	PG	T+PG	PG	T+PG
n = 8	9.8 ± 0.4	8 ± 0.3 <sup>a</sup>	8.5 ± 0.5 <sup>ax</sup>	8.6 ± 0.3 <sup>b</sup>	8.4 ± 0.3 <sup>cx</sup>	8.6 ± 0.2 <sup>b</sup>	9 ± 0.2 <sup>ax</sup>
RBC x 10 <sup>12</sup> /L	158 ± 2	152 ± 3 <sup>a</sup>	155 ± 4 <sup>ax</sup>	154 ± 6 <sup>a</sup>	156 ± 4 <sup>ax</sup>	135 ± 4 <sup>d</sup>	135 ± 7 <sup>cx</sup>
Hb g/L	43 ± 0.5	41 ± 0.8 <sup>a</sup>	41 ± 1 <sup>ax</sup>	42 ± 1 <sup>a</sup>	42 ± 1.7 <sup>ax</sup>	40 ± 1.3 <sup>a</sup>	39 ± 0.7 <sup>dx</sup>
PCV %	1.6	1.9	2.2	3.5	2.5	2.7	3.5
Rt index	13.7 ± 1.1	9.24 ± 0.9 <sup>c</sup>	15.8 ± 1.8 <sup>az</sup>	19.6 ± 2.0 <sup>b</sup>	23.3 ± 3.0 <sup>bx</sup>	17.7 ± 1.9 <sup>a</sup>	19.9 ± 2.3 <sup>bx</sup>

Values are presented as mean ± SE.

Significance: PG and PG+T vs. control: (a) not significant, (b)  $p < 0.05$ , (c)  $p < 0.01$ , (d)  $p < 0.001$

Significance: PG+T vs. PG: (x) not significant, (y)  $p < 0.05$ , (z)  $p < 0.01$

Analysis of rat bone marrow samples revealed that TBMNC were higher in both experimental groups than in group C during the whole course of the experiment (Table 1). The only exception was the T+PG group on the 7<sup>th</sup> day, when TBMNC fell to the control level (Table 1). The bone marrow myeloid : erythroid ratio was 2:1 in group C. During the experiment transition changes in M:E ratio in

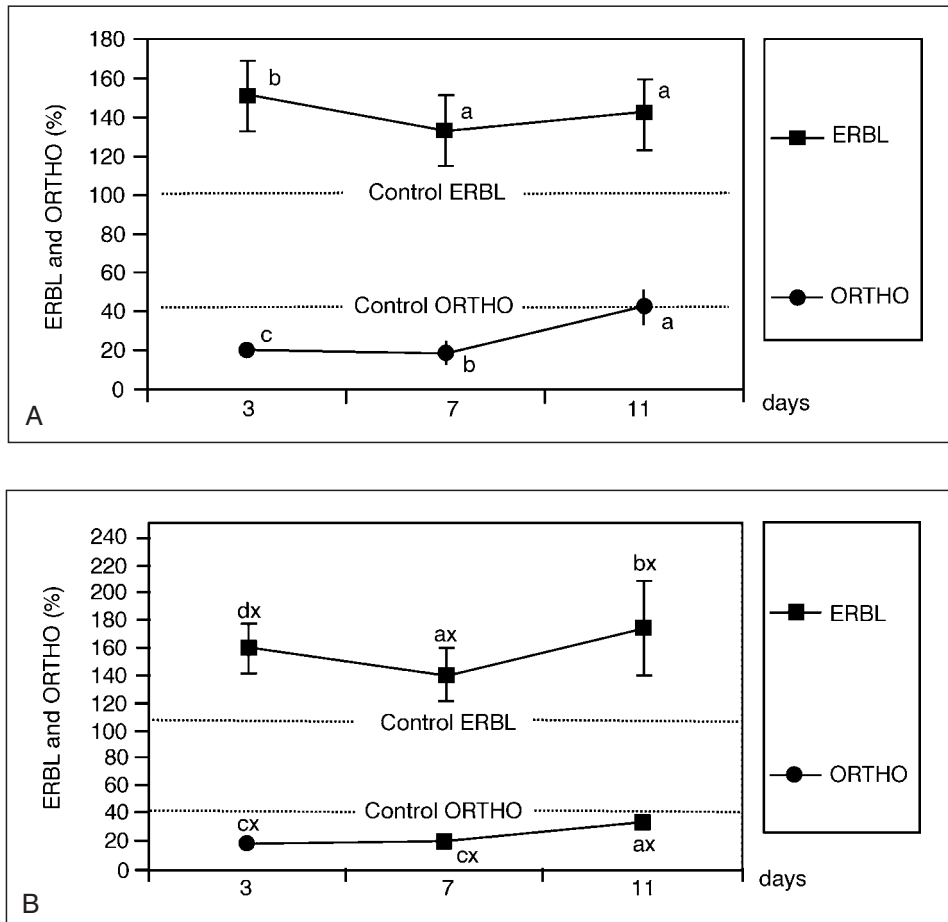


Figure 1. The effect of PG (A) and T+PG (B) application for 3, 7 and 11 days on different bone marrow erythroid precursor cell compartments. Each compartment (ERBL and ORTHO) is presented as a percentage of the total number of nucleated cells per femur (100%).

Data are shown as mean value  $\pm$  standard error. Statistical analysis was performed with the total number of cells.

1. *p* values are expressed vs untreated control rats (punctated line on the figure): (a) not significant, (b)  $p < 0.05$ , (c)  $p < 0.01$ , (d)  $p < 0.001$

2. *p* values are expressed vs PG group of rats: (x) not significant

both experimental groups were observed indicating stimulated erythropoiesis or myelopoiesis (Table 1). From changes in the M:E ratio and elevated number of ERBL, it can be concluded that the high values of TBMNC were a consequence of changes in both myeloid and erythroid cell compartments (Table 1 and Fig. 1). The slight decrease in TBMNC after 3 days (Table 1), correlated with the increase in WBC count in peripheral blood (Table 2).

The number of ERBL was higher in both experimental groups of rats than in group C during the whole course of the experiment, with significantly higher values on the 3<sup>rd</sup> day in group PG and the 3<sup>rd</sup> and 11<sup>th</sup> day in group T+PG (Fig. 1A and 1B). The number of ORTHO significantly decreased on the 3<sup>rd</sup> and 7<sup>th</sup> day in groups PG and T+PG, but both experimental groups returned to control values on the 11<sup>th</sup> day of the experiment (Fig. 1A and 1B).

Analysis of BFU-E number revealed no changes between the control, PG and T+PG groups of rats on the 7<sup>th</sup> day of the experiment, but showed a 3 fold increase in both experimental groups on the 11<sup>th</sup> day (Fig. 2).

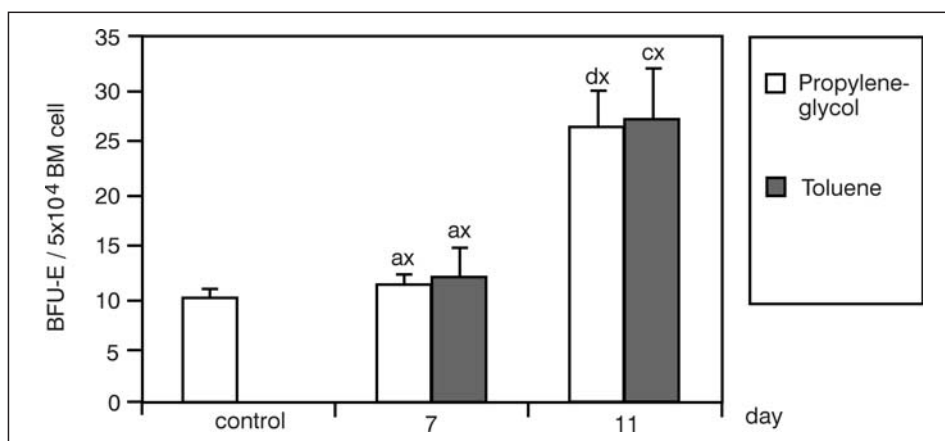


Figure 2. The effect of PG and T+PG application for 7 and 11 days on rat bone marrow BFU-E number. Values are presented as mean  $\pm$  SE.

- $p$  values are expressed vs untreated control rats (punctuated line on the figure):  
 (a) not significant, (b)  $p < 0.05$ , (c)  $p < 0.01$ , (d)  $p < 0.001$
- $p$  values are expressed vs PG group of rats: (x) not significant

According to all examined parameters we concluded that there was no significant difference in bone marrow cellular reaction between the PG and T+PG groups.

#### *Peripheral blood parameters*

In the course of the experiment, PCV decreased slightly, to become statistically significant just on the 11<sup>th</sup> day of the experiment in group T+PG (Table

2). During the whole experiment Hb concentration was lower in both experimental groups than in the control one, but a significant decrease of Hb concentration occurred only after 11 days (Table 2). The RBC number was lower in both experimental groups of rats compared with the control during the whole experiment, the differences being significant after 7 days of application in both experimental groups and on the 11<sup>th</sup> day in group PG (Table 2 and Fig. 3A and

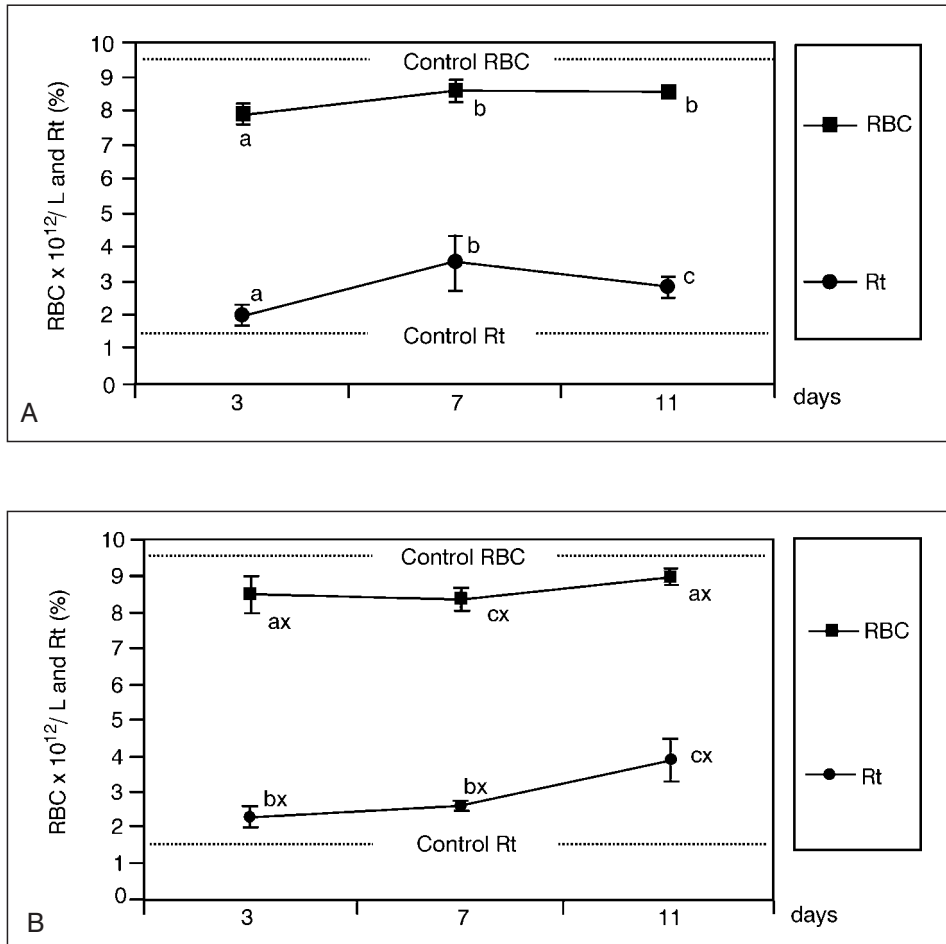


Figure 3. The effect of PG (A) and T+PG (B) application for 3, 7 and 11 days on number of RBC and reticulocyte percentage (Rt) in peripheral blood. Data are shown as mean value  $\pm$  standard error.

1. *p* values are expressed vs untreated control rats (punctated line on the figure): (a) not significant, (b)  $p < 0.05$ , (c)  $p < 0.01$ , (d)  $p < 0.001$
2. *p* values are expressed vs PG group of rats: (x) not significant

3B). During the course of the experiment no significant differences were observed between group PG and T+PG concerning changes in red blood cell parameters. As the percentage of Rt in peripheral blood was higher at all time points than the control value and as the Rt-index remained above 1, we concluded that the bone marrow response in both experimental groups was appropriate (Tab. 2).

#### DISCUSSION

After acute or chronic inhalation or ingestion, toluene is mostly stored in tissues rich in lipids, such as neuronal and bone marrow tissues. Its toxic effects on neuronal tissue are well documented (De Gandarias *et al.*, 1994; Stengard *et al.*, 1994), but a hematotoxic effect has not been evidenced with certainty. The usual route of exposure to toluene is from inhalation of ambient air. However, toxicological data derived from an alternative route of exposure serve to elucidate mechanisms of toxicity. Having a high turnover rate, hematopoietic progenitors and precursor cells are the most susceptible and sensitive to xenobiotic insults. As previously demonstrated (Milenković and Pavlović- Kentera, 1980), increased erythropoietic activity i.e. enhanced cell proliferation and differentiation could be a sensitive indicator of the effects of toxic compounds.

The dose of toluene we used was the daily permitted dose for rats (ACGIH, 1985). In our experimental conditions, during 3, 7 and 11 days of i.p. administration of this toluene dose dissolved in PG or of PG alone, rat red blood cell parameters slightly declined, but anemia did not appear. However, the increased Rt count and reticulocyte index reflected an enhanced and effective erythropoietic activity. Therefore, these findings might be considered suggestive of the compensatory hemolytic state reported after PG exposure (Christopher *et al.*, 1989; Mottu *et al.*, 2001).

During the experiment, the number of erythroid precursor cells changed significantly in both groups, confirming the enhanced erythropoietic activity. At all examined time points the number of ERBL was higher than the control value, but not always significantly. The number of ORTHO decreased significantly on the 3<sup>rd</sup> and 7<sup>th</sup> days of the experiment, probably reflecting shortening of the maturation time and increased release of reticulocytes in to the bloodstream. On the 11<sup>th</sup> day of the experiment the number of ORTHO returned to the control value indicating that a new equilibrium appropriate for the increased demands had been reached. These findings are in accordance with PG induced hypercellularity of bone marrow, due to an increased number of erythroblasts (Christopher *et al.*, 1898). Also, on the 11<sup>th</sup> day of the experiment the number of BFU-E increased significantly. In parallel with the slight decrease in RBC number and enhanced erythropoietic activity, application of PG and T+PG, in our experimental conditions provoked an inflammatory reaction. This inflammatory reaction was confirmed by the increase in number of leukocytes, TBMNC, bone marrow colony forming unit-granulocyte monocyte and the concentration of ceruloplasmin (Božić *et al.*, 2003). Thus, stimulated erythropoiesis could partially reflect the effect of a concomitantly present inflammatory reaction. Namely, stimulated



erythropoiesis was also demonstrated in polivinylpyrrolidol induced acute sterile inflammation in rats (Petakov *et al.*, 1998).

#### CONCLUSION

As there were no differences in the examined parameters between the T+PG and PG groups, our results indicate that short-term intraperitoneal application of 3mg/kg of toluene, does not have a toxic effect on rat erythropoiesis stimulated by the hemolytic effects of propylene glycol.

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## UTICAJ INTRAPERITONEALNE APLIKACIJE TOLUENA RASTVORENOG U PROPILEN GLIKOLU NA ERITROPOEZU PACOVA WISTAR SOJA

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### SADRŽAJ

U ovom radu su prikazani rezultati ispitivanja mogućeg toksičnog uticaja toluena na eritropoezu tokom pojačane eritropoetske aktivnosti, izazvane aplikacijom blagog hemolitičkog agensa propilen glikola, kod odraslih ženki pacova Wistar soja. Eksperimentalnim životinjama je jedanput dnevno, tokom 3, 7 i 11

dana, intraperitonealno aplikovan toluen rastvoren u propilen glikolu (T+PG), odnosno samo propilen glikol (PG). Efekti T+PG i PG na hematološke parametre periferne krvi i kostne srži su određivani u vreme žrtvovanja životinja.

Broj eritrocita, retikulocita, hematokritska vrednost i koncentracija hemoglobina su određivani u perifernoj krvi standardnim laboratorijskim procedurama. U kostnoj srži je određivan ukupan broj ćelija sa jedrom i ispitan odnos ćelija prekursora mijeloidne i eritroidne loze. Broj ranih opredeljenih matičnih ćelija za eritrocitopoezu (BFU-E) određen je *in vitro*, na osnovu broja kolonija formiranih iz ćelija kostne srži na polučvrstoj podlozi od metil celuloze.

Toluen rastvoren u propilen glikolu je u perifernoj krvi izazvao pad broja eritrocita, pad hematokritske vrednosti, smanjenje koncentracije hemoglobina, a doveo je do porasta udela retikulocita. U kostnoj srži je došlo do porasta broja BFU-E, kao i eritrocitnih prekursora, što ukazuje na pojačanu eritropoetsku aktivnost. Ispitivani parametri se nisu značajno razlikovali prilikom aplikacije samog propilen glikola u odnosu na vrednosti parametara dobijenih prilikom aplikacije toluena rastvorenog u propilen glikolu. Zbog toga možemo da pretpostavimo da kratkotrajna aplikacija niske doze toluena (3 mg/kg) nema toksičan efekat na eritropoezu tokom pojačane eritropoetske aktivnosti izazvane aplikacijom propilen glikola.