MORPHOLOGY OF THE SPLEEN IN ADULT ALBINO RATS AFTER WHOLE-BODY EXPOSURE TO LOW-LEVEL OF TOLUENE

Vladimir N. Voloshin *1, Vladimir G. Koveshnikov 2, Irina S. Voloshina 3.
*1,2 Professor, 3 Associate Professor.
Department of Anatomy, Lugansk State Medical University, Ukraine.

ABSTRACT

Background: Spleen is a secondary lymphoid organ that is highly sensitive to different chemicals. Toluene is an aromatic hydrocarbon commonly used as an industrial solvent and can be considered as a potential immunotoxin.

Aims and Objects: We aimed to investigate the organometry and histology of spleen from rats exposed to toluene.

Materials and Methods: The toxicity of toluene was evaluated in male albino rats (6/group) via whole-body exposure. The animals were exposed to target concentrations of 0 (air control) and 133 parts per million (ppm) of toluene in air for 5 hours/day, 5 days/week, for 2 month. The animals were weighted and decapitated at different time points (one, seven, sixteen, thirty one and sixty one day) post-exposure. The weight, length, width and thickness of the spleen were measured. It was studied absolute and relative weight of the spleen. Histological examination of the spleen was made by light microscope.

Results: Statistically significant difference between the mean of body weight from the control and experimental animals was observed seven days after last exposure to toluene. Thus, the body weight of animals exposed to toluene was 254.17 g, that was 7.18% (p = 0.048) below control data. The histological findings showed increased area of the white pulp of spleen from rats exposed to toluene had increases compared to that from the control. The numbers of siderophages were higher in the spleen from rats exposed to toluene. The relative area of germinal centre in the structure of the splenic lymph follicles of rats exposed to toluene increased. We found that in the first and second experimental groups of animals the indication was at the level 10.86% and 10.26% respectively. These are 24.11% (p = 0.002) and 26.04% (p <0.001) above control data, respectively. Inhalation exposures to toluene vapor at 133 ppm produces hyperplasia of lymphoid tissue of the rat spleen.

Conclusion: Our data demonstrated that whole-body exposures to low-level of toluene led to hypertrophy of white pulp of the spleen.

KEYWORDS: Toluene; Toxicity; Rats; Spleen; Histology.

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INTRODUCTION

Immunotoxicology deals with the study of the potential effects of xenobiotics on the immune system. An immunotoxic compound is defined as a compound that can alter one or more immune functions resulting in an adverse effect. The immune system is a very complex and regulated system [1] that involves the cooperation and interaction of a number of different cell types, cell products, tissues, and organs. The immune system consists of fixed primary (i.e., thymus and bone marrow) and secondary (i.e., spleen, lymph nodes, and gut-associated) lymphoid tissue, and various circulating immunocompetent cells. This unique organization may contribute to the immune system's vulnerability as a target organ for xenobiotics. For example, cells of the
immune system undergo continual proliferation and differentiation for self-renewal to maintain immunocompetence and thus can be affected by xenobiotics that alter this cellular balance. In addition, data indicate that immune responses can be regulated by other organ systems such as the nervous and endocrine systems [2, 3]. Although the immune system is not typically considered as an organ primarily involved in the absorption or metabolism of xenobiotics, parts of the immune system (e.g., gut-associated lymphoid tissue) receive significant initial exposure after ingestion and immune cells have been reported to metabolically activate xenobiotics [4], and thus along with the liver, may play an important role in mediating a particular metabolizing effect on immunocompetence.

Several animal studies have identified a number of chemicals with a potential to cause immunosuppression. These chemicals are commonly used in the workplace such as pesticides, metals, and industrial chemicals [5]. The possibility that environmental pollutants may interfere with immune functions in humans has been raised in a growing number of studies focusing on subjects accidentally or occupationally exposed to industrial or environmental chemicals [6].

Toluene (methylbenzene, toluol, phenylmethane) is an aromatic hydrocarbon commonly used as an industrial solvent for the manufacturing of paints, chemicals, pharmaceuticals, and rubber. It is identified as CAS#108-88-3, and the United Nations Department of Transportation’s number for toluene is UN#1294. Toluene is found in gasoline, acrylic paints, varnishes, lacquers, paint thinners, adhesives, glues, rubber cement, airplane glue, and shoe polish. At room temperature, toluene is a colorless, sweet smelling, and volatile liquid [7].

Toxicity can occur from unintentional or deliberate inhalation of fumes, ingestion, or transdermal absorption. Toluene abuse or “glue sniffing” has become widespread, especially among children or adolescents, because it is readily available and inexpensive. Toluene is commonly abused by saturating or soaking a sock or rag with spray paint, placing it over the nose and mouth, and inhaling to get a sensation of euphoria, buzz, or high. Slang names for inhalation include huffing (i.e., soaking a sock or rag) and bagging (i.e., spraying paint into a plastic bag and inhaling). With bagging, exhaled air is rebreathed and resulting hypoxia and hypercarbia may add to the disorienting effects of the solvent [8].

The Occupational Safety and Health Administration (OSHA) has determined the acceptable level of occupational exposure to toluene for people in the workplace. The Permissible Exposure Limit (PEL) of 200 ppm is considered an acceptable level of exposure as a time-based average for an 8-hour workday [9]. Toluene levels of 500 ppm are considered immediately dangerous to life and health. Though the role of the global and occupational environment in the development of lymphoid organs reactions is obvious [10], morphology of the rats spleen under influence of toluene is unclear. Thus, the present study was undertaken to study the gross anatomical and histological alterations of the spleen of albino rats exposed to toluene.

MATERIALS AND METHODS

Experimental animals

Sixty adult male laboratory albino rats (Rattus norvegicus) were obtained from Lugansk State Medical University Laboratories (Lugansk, Ukraine). When received, the rats were 88-92 days of age and weighed 130-150 g. Rats were maintained (6/cage) in polycarbonate cages with hardwood bedding. Stainless steel wire mesh cages were used in the exposure chambers. Temperatures were maintained at 22°C ±3°C, with a relative humidity of 40-60%. Lighting was timer-controlled to provide a 12-h light-dark cycle, with light onset at 7:00 a.m. Laboratory Rodent Diet 5001 (PMI Feeds, Ukraine) was used. Body weights were measured daily. Food allotments were given at the end of the day. Tap water was provided ad libitum with water bottles. For the 5-h periods in the exposure chambers, rats did not receive food or water.

The experiments were performed according to the Guidelines for Animal Experiments prepared by the Committee for the Welfare of Experimental
Animals in Lugansk State Medical University.

Experimental Design

Five treatment groups, each with six rats, were used in each of two replicates; one used as control rats (A-series) and the other used as toluene-exposed rats (B-series). The B-series rats were exposed to 133 ppm toluene for 5 h/day 5 days/week. The total number of exposures is sixty. Control rats inhaled clean air and have been exposed to shame reaction for similar time.

Test Chemical

The whole-body exposure to toluene is the typical route of administration in most human cases. Certified ACS (99.9% pure) toluene (CAS Number 108-88-3) obtained from Macrochem (Kiev, Ukraine) was used.

Exposure Chambers

Laboratory quantities of toluene were kept in tightly closed brown glass jugs placed in protective plastic enclosures. When not in use, they were stored in a fire-proof cabinet located in a cool, well-ventilated area away from oxidizing agents and sources of heat or ignition. The air exhaust system in the inhalation facility operated at negative pressure from the point where the solvent entered the air stream until the air was exhausted from the building. This ensured that any leaks in the system resulted in fresh air being drawn into the ducting rather than allowing solvent vapors to escape into the laboratory. The exposure laboratory also had a negative pressure exhaust system with an air inlet near floor level for collection of vapors heavier than air.

The two 1.0 m$^3$ exposure chambers were constructed of glass and stainless steel and were operated at a flow rate sufficient to ensure 12-15 conditioned, filtered air changes per hour. A positive-displacement flowmeter located on the inlet side of each chamber monitored the airflow rate. This flow was displayed and recorded every five minutes. Chamber temperature also was displayed and recorded every five minutes.

The test atmosphere generation system was designed specifically for solvents. Conditioned input air passed through an in-line heating unit that dispensed vaporized toluene into the airstream. Toluene was introduced into the evaporator by adjustable pumps; the type of pump and flow rate depended upon the toluene concentration being generated. A microprocessor-based temperature controller heated the evaporators. The vapor was delivered through a stainless steel duct system to the animal chamber inlets.

Actual toluene concentrations for exposure groups were determined by monitoring the chamber atmospheres using a dedicated M200 gas chromatography system. The gas chromatograph readings were stored in a computer for analysis. Air from chamber was sampled every fifteen minutes.

Exposures

B-series groups of animals (133 ppm) were placed in one chamber and the remaining A-series animals were placed in a second chamber (0 ppm). Once the animals of B-series were placed in the exposure chambers, it took about fifteen minutes for the toluene to reach the desired concentration. After 5 h, the toluene supply was turned off; it then took about fifteen minutes for the toluene concentration to drop to near 0 mg/m$^3$. One (1st group), seven (2nd group), sixteen (3rd group), thirty one (4th group) and sixty one (5th group) days after the last exposure to toluene, rats were weighed and decapitated. Spleens were weighted and were harvested from control and B-series rats.

Organometry

Spleens were removed from decapitated rats, weighed and photographed using Video Presenter SVP-5500 (Samsung Techwin Co. LTD, Korea) and the scale. Using the software ImageJ 1.46r (Wayne Rasband National Institutes of Health, USA; http://rsb.info.nih.gov/ij/) the length, width and thickness of the spleen were measured.

Histopathological examination by light microscope

Spleens from toluene exposed and control rats were fixed in 4% paraformaldehyde in 0.01 M PBS. After fixation, a cross-section from the middle region of the spleen was embedded in paraffin wax. A section from each paraffin block was stained with haematoxylin and eosin and with Pearl’s Prussian blue staining for histopath-
ological evaluation by light microscope Olympus BX-41 (Olympus Corporation, Japan).

**Statistical analysis**

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by a post hoc test. The distribution of data was checked for normality by the Kolmogorov–Smirnov test. Numeric data for each A-series p value of <0.05 was regarded as significant. Statistical analysis was performed by the Statistica software (StatSoft, Inc., USA; http://www.statsoft.com).

**RESULTS**

Body weight of animals after whole-body exposure to toluene

The mean body weight of the 1st group of B-series rats was 249.83 g that is 5.72% (p = 0.042) lower than the same indicator in the corresponding control group rats. Statistically significant difference between the mean body weight were also observed in control and experimental animals of the 2nd group. Thus, the body weight of animals exposed to toluene was 254.17 g, that is 7.18% (p = 0.048) below control data (Table 1).

<table>
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<th>Final body weight (g)</th>
<th>Weight gain (g)</th>
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<td>130</td>
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Table 1: Indicators of body weight control (A-series) and rats exposed to toluene (B-series).

* statistically significant difference.

**Absolute and relative weight of the spleen of rats exposed to toluene**

The absolute weight of spleen from rats exposed to toluene of 1st group was above the control data at 1.11% (p = 0.567). The mean data of this indication in 2nd group of experimental animals almost did not differ (p = 0.239) from the control data. It was 804.67 mg. Statistically significant difference between the mean of absolute spleen weight in A- and B-series animals was in the 3rd group.

The absolute weight of the spleen of 4th and 5th groups of B-series animals were not statistically different from the control parameters (p> 0.05) and were 812.50 mg and 838.17 mg, respectively.

The relative weight of spleen of 1st group rats exposed to toluene was higher than the control data at 7.59% (p = 0.011). This indication in the rats of 2nd experimental group was set at 317.90 mg/100 g body weight. In 3rd group rats mean data of relative spleen weight was above the control data at 7.56% (p = 0.085), and in the 4th and 5th groups was lower than the control at 6.06% (p = 0.037) and 1.16% (p = 0.496) respectively (Fig. 1).

**Size of the rats’ spleen exposed to toluene**

In the 1st group of animals exposed to toluene, mean spleen length was 37.81 mm, that is 4.37% (p = 0.094) below control data. There was no significant difference between control and experimental data in the 2nd groups of animals. Thus, in the rats exposed to toluene the length was lower than control at 0.17 mm (p = 0.847).

Only in the 3rd group of animals the mean data of the spleen length was lower than control at 4.71% (p = 0.049) (Fig. 2).

The width of the spleen in rats of 1st and 2nd groups of rats exposed to toluene was lower than the control data. However, this difference was statistically unreliable. Thus, in the 1st group it was 1.44% (p = 0.668), and in the 2nd – 1.49% (p = 0.662). The most statistically significant difference between the spleen width was obse-
erved in animals that were removed from the experiment 30 days after termination of toluene exposure ($p = 0.060$) (Fig. 3).

The thickness of the spleen of 1st experimental rats group was 4.47 mm, that at 0.11 mm, or 2.40% ($p = 0.326$) was lower than the control. In 2nd group of B-series animals the indication was greater than control data at 0.10 mm ($p = 0.581$).

Thickness of the spleen of experimental rats that were removed from the experiment 15 days after termination of toluene exposure was 4.30 mm. It was 0.05 mm ($p = 0.722$) above the control data. The mean data of the indication in 4th and 5th groups of animals were 3.03% ($p = 0.432$) and 2.39% ($p = 0.391$) lower than the control, respectively.

Fig. 1: Relative weight of the spleen from control animals (A-series) and from rats exposed to toluene (B-series) after the last exposure. Relative weight of the spleen from rats of B-series was significantly increased (1st group) and decreased (4th group). Each group included 6 animals. *$p <0.05$.

Fig. 2: (A) The spleen from control rat sixteen days after the last exposure showing a normal size. (B) The spleen from rat exposed to toluene sixteen days after the last exposure.

Fig. 3: Length of the spleen from control animals (A-series) and from rats exposed to toluene (B-series) after the last exposure. The indicator was decreased in rats of B-series. This decreasing was significant in the 3rd group of animals. Each group included 6 animals. *$p <0.05$. 

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**Fig. 4:** (A) The spleen of 1<sup>st</sup> group control rat (low magnification). Normal histology of rat spleen. (B) The spleen of 1<sup>st</sup> group rat exposed to toluene (low magnification). Increase of white pulp area. LF – lymphoid follicle; PALS – periarterial lymphoid sheath; RP – red pulp; WP – white pulp; * – area of extramedullary haemopoiesis; ** – trabecular artery; arrow shows central artery. H&E.

**Fig. 5:** (A) The spleen of 3<sup>rd</sup> group control rat. (B) The spleen of 3<sup>rd</sup> group rat exposed to toluene. Region of the marginal zone and red pulp. There is increasing number of the siderophages in the spleen from rat exposed to toluene. Pearl’s Prussian blue.

**Fig. 6:** Level of the white pulp of the spleen from control animals (A-series) and from rats exposed to toluene (B-series) after the last exposure. The level was increased significantly in rats of B-series. *p < 0.05; **p < 0.001.

**Fig. 7:** (A) The spleen of 2<sup>nd</sup> group control rat showing normal histology (high magnification). Region of the periarterial lymphoid sheath. (B) The spleen of 2<sup>nd</sup> group rat exposed to toluene (high magnification). Region of the lymphoid follicle. There is increasing compartments of the white pulp. CA – central artery; GC – germinal centre; MnZ – mantle zone; MS – marginal sinus; MZ – marginal zone; PALS – periarterial lymphoid sheath; RP – red pulp. H&E.
Histopathology of the spleen of rats exposed to toluene

At low magnification we found that area of the white pulp of rats exposed to toluene increases compared to the levels in the control. It was noted that region of the periarterial lymphoid sheath in the animals of B-series increased (Fig. 4). Number of siderophags in the rats exposed to toluene increased (Fig. 5) compared with control.

Rats that were removed from the experiment after 1 and 7 days after last exposure to toluene, had a relative area of white pulp at 51.83% and 63.46%, that are 21.52% (p = 0.023) and 34.94% (p <0.001) above that of controls. In animals of 3rd and 4th groups this difference was registered at 24.72% (p <0.001) and 24.54% (p = 0.004), respectively (Fig. 6).

The relative area of germinal centre in the structure of the splenic lymph follicles of rats exposed to toluene increased (Fig. 7). We found that in the 1st, 2nd and 3rd experimental groups of animals the indication was at the level 10.86%, 10.26% and 9.45% respectively. These are 24.11% (p = 0.002), 26.04% (p <0.001) and 34.42% (p = 0.001) above control data, respectively. After 30 and 60 days after termination of toluene exposure relative area of the germinal centre was already 8.74% and 9.37%, which was higher than the control at 16.22% (p = 0.007) and 20.75% (p <0.001), respectively.

The relative area of mantle zone of lymph follicles of 1st and 2nd groups rats exposed to toluene were 28.16% and 32.88%, that is below the control data at 30.43% (p = 0.026) and 26.34% (P <0.001), respectively. After day 1 and 60 following toluene exposure the relative area of marginal zone of lymph follicles was at 60.98% and 57.25%, that are 20.11% (p = 0.046) and 16.77% (p = 0.006) above the control data.

DISCUSSION

Toluene is among the ingredients of various materials that humans can face during daily life, such as paints, paint thinners, adhesives, fingernail polish and gasoline. In addition, there are increased risk groups that are exposed to toluene occupationally. In this study, we measured the effect of toluene on anatomy of the spleen of albino rats.

Currently there are no previous studies that have shown the effects of toluene exposure on rodent spleen morphology. In previous studies, the significant effect of low concentration of toluene on the immune system organs [11] has not shown. It was indicated a decrease in thymus weight when exposed to high concentrations of the reagent [12]. So, to our knowledge, this is the first study examining the effect of toluene exposure in low concentration (133 ppm) on the size and histology of spleen in rats.

Although spleen weight and histology are routinely examined in conventional toxicity studies, examples of significant changes are very few. Spleen weight is a relatively insensitive correlate of immunosuppression [13]. In most instances, spleen atrophy, especially in T cell areas, is associated with thymus atrophy [14].

Over the last several years, the histomorphologic assessment of the immune system has moved to the forefront of the tools for identifying immunotoxicity. Numerous scientific forums have attempted to address the sensitivity, specificity, and consistency of histopathology for identifying immunotoxicity risks of new chemical entities. Suggestions for advanced pathology training and harmonization of terminology have been proposed, and a “Best Practices” paper, along with an extensive monograph on the subject has been published [15, 16], each with the intent of providing histopathologists with the tools necessary to accurately and consistently characterize alterations of the immune system [17, 18].

The present results about body weight of animals exposed to toluene confirmed earlier studies. Thus, it was reported that final body weights of Fischer 344 rats exposed to toluene vapors at 2500 and 3000 ppm (9422 and 11,307 mg/m³, respectively) for 6.5 hours/day, 5 days/week for 15 weeks [19] were 15 and 25% lower in the males and 15 and 14% lower in the females of the 2500- and 3000-ppm groups compared to the controls, respectively. Although, Von Euler et al. [20] exposed 30 male Sprague-Dawley rats
to 80 ppm (302 mg/m$^3$) toluene for 6 hours/day, 4 days/week for 4 weeks and shown no effects on body weight were reported. Similar results were shown on B6C3F1 mice exposed to 0, 120, 600, or 1200 ppm of toluene 6.5 hours/day, 5 days/week by Huff [21].

Tin-Tin Win-Shwe et al. [22] investigated the effects of low-level toluene exposure on immunological biomarkers. They found that splenic T lymphocyte subsets and mRNA expression levels of Th1 cytokine IL-12, transcription factor T-bet, and Foxp3 were significantly suppressed in PND 21 male mice exposed to toluene. To investigate the effect of low-level toluene inhalation on immune regulation in an allergic mouse model, C3H/HeN mice were exposed to 0, 5, 50, or 500 ppm of toluene for 6 h/day, 5 days/week for 3 or 6 weeks. Exposure to 500 ppm significantly increased the expression of transcription factors STAT3, STAT4 and STAT5a mRNAs in spleen [23]. Other findings suggest that low-level toluene exposure and PGN stimulation from the late prenatal to early postnatal stage suppressed the splenic parameter related to Th1/Th2 immunity in infant mice [24].

To date there is no literature data about the changes in shape and spleen histology of animals exposed to toluene inhalation. In the current investigation we demonstrated that toluene treatment led to white pulp hyperplasia. The relative area of marginal zone and relative area of germinal centers of lymph follicles have increased. Our data coincide with the results of other authors, who observed hyperplasia of lymphoid tissue following exposure to certain toxic substances in monkeys [25] and rats [26].

We assume that toluene has an indirect effect on the structure of the spleen via disturbing the function of nervous system. Toluene is highly lipophilic, which accounts for its primary effects on the central nervous system (CNS). After crossing the blood-brain barrier, toluene, along with other volatile anesthetic agents, had been previously thought to inhibit neuronal transmission by causing a change in membrane or membrane protein conformation. Recent research has shown that interactions with several key brain neurotransmitters (i.e., Gama-Amino Butyric Acid (GABA), glycine, glutamate, acetylcholine, dopamine) are responsible for the clinical effects seen [27]. Postmortem studies along with magnetic resonance imaging (MRI) findings have shown diffuse white matter demyelination and gliosis (solvent vapor/toluene leukoencephalopathy), which is postulated to be the end product by which chronic toxicity occurs, although the exact mechanism by which this occurs remains unclear [28].

Nerve fibers containing neuropeptides and neurotransmitters are observed in various lymphoid tissues where they directly come in contact with immune cells such as lymphocytes. Conversely, products of the immune system (i.e., cytokines) have been reported to affect neuroendocrine functions [29]. Additionally, various hormone receptors have been found on immune cells and a number of hormones have been reported to enhance (e.g., growth hormone, thyroid stimulating hormone, and prolactin), attenuate (e.g., gonadal steroids and endogenous opioids), or suppress (e.g., glucocorticoids and adrenocorticotropin) responses of the immune system [30]. Immune cells have also been reported to produce various peptide and protein hormones such as growth hormone [31], prolactin [32], luteinizing hormone, thyrotropin-stimulating hormone, and adrenocorticotropic [33]. Immune cell function is altered following the exogenous addition of neurotransmitters, neuropeptides, or cytokines in vitro. Thus, if a xenobiotic found in the environment alters the production or release of neurotransmitters and neuropeptides, it may also alter the function of the immune system. In addition, number of haemosiderin-bearing cells in the spleen under these conditions increases. This is probably due to increased levels of destruction of red blood cells in the pulp [34]. The data regarding stress-induced changes in the spleen, as to increase the represented by the increased area of the white pulp and its compartments, were reported by other authors who have noted increasing proliferation of lymphoid tissue in the peripheral organs of the immune system under conditions of stress factors [35]. Since toluene is a widely used material and there are many people exposed to it, toluene exposure becomes a major public health problem making the results
of this study important. Future studies on toluene exposure should focus on clarifying organometry and histology of thymus as central lymphoid organ.

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