

Spleen Organometry of Old Male Albino Rats After Whole-Body Exposure to Toluene

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Abstract

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. We aimed to investigate the organometry of the spleen from rats exposed to toluene. The toxicity of toluene was evaluated in male albino rats via whole-body exposure. The animals were exposed to target concentrations of 0 (air control) and 133 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.

Rats that were withdrawn from the experiment 30 days after the cessation of toluene had an organ weight of 1134.67 mg. This indicator was not statistically significantly higher than the data of the respective control group by 5.75% ($p = 0.109$). The weight of the organ of rats of group 5 of series T was at the level of 1217.33 mg, which was 103.11% ($p = 0.222$) to the control. Rats that were withdrawn from the experiment 7 and 15 an organ length of 41.64 mm and 43.04 mm, respectively. In the 4 and 5 groups of rats, the average length of the organ was 104.71% ($p = 0.076$) and 97.82% ($p = 0.282$) to the data of the corresponding groups of the control series. Rats withdrawn from the experiment 15 and 30 days after toluene cessation had an organ thickness of 4.91 mm and 4.97 mm, which was 100.40% ($p = 0.729$) and 103.11% ($p = 0.927$) to the control indicators. The results demonstrate that inhaled toluene does not significantly affect the weight and size of the spleen of old rats.

Keywords: Toluene; Toxicity; Rat; Spleen

Introduction

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 (thymus and bone marrow), secondary (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 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. 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 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 rat 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

Sixty old male laboratory albino rats were obtained from St. Luke Lugansk State Medical University Laboratories. When received, the rats were 88 - 92 days of age and weighed 300 - 330g. 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 $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$, 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 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 St. Luke Lugansk State Medical University.

Five treatment groups, each with six rats, were used in each of two replicates; one used as control rats (C-series) and the other used as toluene exposed rats (T-series). The T-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.

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) was used. 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 1.0m³ 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.

T-series groups of animals (133 ppm) were placed in one chamber and the remaining C-series animals were placed in a second chamber (0 ppm). Once the animals of T-series were placed in the exposure chambers, it took about fifteen minutes for the toluene to reach the desired concentration. After 5h, the toluene supply was turned off; it then took about fifteen minutes for the toluene concentration to drop to near 0 mg/m³. 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 T-series rats.

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 ImageJ1.46r (Wayne Rasband National Institutes of Health, USA; <http://rsb.info.nih.gov/ij/>) the length, width and thickness of the spleen were measured. 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

The average body weight of rats in group 1 exposed to toluene was 366.67g, while the data of the control group was at the level of 373.33g, which is 1.78% ($p = 0,557$) above the values of the series C. Rats that were withdrawn from the experiment 7 days after cessation of toluene had a body weight of 362.50g. This parameter was 98.46% ($p = 0.718$) to the value of the corresponding control group. The maximum value of this indicator in rats of 3 group T series was 395g and the minimum - 331g. The average was 361.00g, which is 2.65% ($p = 0.494$) below the control values. Rats withdrawn from the experiment 30 and 60 days after toluene cessation had a body weight of 386.17g and 383.33g, respectively, which was 102.97% ($p = 0.256$) and 103.84% ($p = 0,275$) to the indicators of the control series. The increase in body weight in the last groups of animals of the first series amounted to 45.00g (13.19%) and 40.66% (11.87%).

The absolute weight of the spleens of rats of group 1 of series T was fixed at 1233,50 mg, which is 7,25% ($p = 0,030$) above the values of the corresponding control group and allows to confirm the statistical probability of the difference between the mean values of these groups. Rats that were withdrawn from the experiment 7 days after the cessation of toluene had an average spleen weight of 1070.83 mg, which was 102.16% ($p = 0.446$). The maximum value of organ weight in rats of group 3 was at the level of 1393g and the minimum - 1254g. The mean value of the absolute weight of the spleen was 1316.67 mg, which was above the control values by 6.77% ($p = 0.078$). Rats that were withdrawn from the experiment 30 days after the cessation of toluene had an organ weight of 1134.67 mg. This indicator was not statistically significantly higher than the data of the respective control group by 5.75% ($p = 0.109$). The weight of the organ of rats of group 5 of series T was at the level of 1217.33 mg, which was 103.11% ($p = 0.222$) before control (Table 1).

The mean of the relative weight of the spleen in rats of group 1, which were exposed to toluene, statistically significantly exceeded the 9.56% ($p = 0,041$) and was 337.60 mg/100g. The highest was 375 mg/100g and the lowest - 300.52 mg/100g. The rats that were withdrawn from the experiment 7 days after the cessation of toluene had a relative body weight of 279.12 mg/100g, 3.98% ($p = 0.470$) more than the corresponding control group. In the 3 and 4 groups of rats, this indicator was found to be 267.29 mg/100g and 294.16 mg/100g, respectively. This amounted to 80.32% ($p = 0.095$) and 102.83% ($p = 0.338$) to the control indicators. The maximum value of the relative weight of the spleen in the 5 group of animals exposed to toluene was recorded at 351.08 mg/100g and the minimum - at 289.25 mg/100g. The average value of the relative weight of the spleen was 318, 67 mg/100g, which is 0.63% ($p = 0.890$) below control (Table 1).

| Series | Groups | Absolute weight (mg) | | Relative weight (mg/100g body weight) | |
|---------|--------|----------------------|-------|---------------------------------------|-------|
| | | Mean | SD | Mean | SD |
| Control | 1 | 1150,17 | 63,75 | 308,15 | 9,82 |
| | 2 | 1048,17 | 54,37 | 285,74 | 26,66 |
| | 3 | 1233,17 | 88,62 | 332,77 | 21,88 |
| | 4 | 1073,00 | 62,73 | 286,06 | 3,44 |
| | 5 | 1180,67 | 40,03 | 320,68 | 22,90 |
| Toluene | 1 | 1233,50* | 49,47 | 337,60* | 29,06 |
| | 2 | 1070,83 | 44,11 | 297,12 | 25,75 |
| | 3 | 1316,67 | 54,44 | 267,29 | 40,30 |
| | 4 | 1134,67 | 58,74 | 294,16 | 19,43 |
| | 5 | 1217,33 | 56,07 | 318,67 | 25,92 |

Table 1: Absolute and relative weight of the spleen of control rats and animals exposed to toluene.

In one group of animals of series T, the length of the organ slightly exceeded the indicators of the control series (Table 2). This difference was 3.24% ($p = 0.114$). Rats that were withdrawn from the experiment 7 and 15 an organ length of 41.64 mm and 43.04 mm, respectively. In the 4 and 5 groups of rats, the average length of the organ was 104.71% ($p = 0.076$) and 97.82% ($p = 0.282$) to the data of the corresponding groups of the control series. The width of the spleen of rats exposed to toluene in all groups exceeded the values of the control series. However, the difference with the control data was statistically insignificant. Thus, in 1 and 2 groups of animals, the width of the spleen was 8.65 mm and 9.02 mm, which was 102.25% ($p = 0.622$) and 101.69% ($p = 0.545$) to the indicators of the respective groups of the control series. Rats withdrawn from the experiment 30 and 60 days after toluene cessation had an organ width of 9.27 mm and 10.14 mm, respectively. The average index of the thickness of the spleens of rats of group 1 of the series T was 4.85 mm, which is 3.85% ($p = 0.357$) above the control values. Rats withdrawn from the experiment 15 and 30 days after toluene cessation had an organ thickness of 4.91 mm and 4.97 mm, which was 100.40% ($p = 0.729$) and 103.11% ($p = 0.927$) to the control indicators (Table 2).

| Series | Groups | Length (mm) | | Width (mm) | | Thickness (mm) | |
|---------|--------|-------------|------|------------|------|----------------|------|
| | | Mean | SD | Mean | SD | Mean | SD |
| Control | 1 | 39,78 | 1,34 | 8,46 | 0,69 | 4,67 | 0,37 |
| | 2 | 42,96 | 1,53 | 8,87 | 0,52 | 4,79 | 0,30 |
| | 3 | 41,85 | 1,14 | 8,63 | 0,65 | 4,83 | 0,43 |
| | 4 | 42,22 | 1,65 | 9,05 | 0,64 | 4,95 | 0,34 |
| | 5 | 42,70 | 1,71 | 8,94 | 0,64 | 4,81 | 0,29 |
| Toluene | 1 | 41,07 | 1,25 | 8,65 | 0,61 | 4,85 | 0,27 |
| | 2 | 41,64 | 1,21 | 9,02 | 0,34 | 4,83 | 0,28 |
| | 3 | 43,04 | 2,38 | 8,79 | 0,67 | 4,91 | 0,31 |
| | 4 | 44,21 | 1,83 | 9,27 | 0,43 | 4,97 | 0,28 |
| | 5 | 41,77 | 1,04 | 10,14 | 0,51 | 4,96 | 0,33 |

Table 2: Sizes of the spleen of control rats and animals exposed to toluene.

Discussion and Conclusion

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 been 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/m³) 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 adrenocorticotropin [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. 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. 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.

Conflicts of Interests

The authors declare that there is no conflict of interest.

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