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The immunotoxicological pattern of subchronic and chronic benzene exposure in rats

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ABSTRACT

Exposure to benzene and its inevitable metabolites can result in deleterious effects on human health, including lymphocytopenia, hematotoxicity and cancer. However, the duration of exposure might alter the effects including immune consequences. The aim of this study was to determine whether benzene could modulate lymphocyte proliferation induced by the T cell mitogen concanavalin A, in rats, at different exposure durations. 386 Wistar rats were assigned into control and treatment groups which were subdivided into groups for 45, 90 and 135 days for 0,6 mL/kg of drinking water mixed benzene treatment. The percentage of CD3+, CD4+, CD8+ spleen lymphocytes was defined using the flow cytometer. Interleukin (IL)-4, IL-6, IL-10 and interferon-gamma, in supernatants of splenocyte cultures stimulated with Concanavalin A, were assessed by enzyme-linked immunosorbent assay (ELISA) technique. The decrease in the total lymphocyte and T cell counts were associated with increased benzene exposure duration. Th2-type cytokine, IL-4 significantly increased, whereas IL-6, CD4 + T cells, CD4+/CD8+ ratio and CD3+ T cells decreased. Despite the positive correlation between benzene toxicity and indicated increased immune responses, 45-day exposure to benzene appeared to be the most sensitive time point for evaluating benzene cytotoxicity.

1. Introduction

Benzene is an aromatic hydrocarbon that is both anthropogenically produced also occurs naturally. In addition to air, it can be found as a contaminant in soil, drinking water and food. Therefore, human can frequently exposed to benzene via several sources, including smoking, exhaust gasses, emissions of industrial, petrochemical and pharmaceutical production processes. Despite its known multiorgan interferences, the sensitive populations, such as children, pregnant women, elderly, immunocompromised individuals and occupational workers are at risk for adverse health effects because of the elevated atmospheric concentrations of benzene (Rich and Orimoloye, 2016). Long term exposure to benzene can cause acute myeloid leukemia (AML) and has effects in the bone marrow, decreasing the red blood cells and other components, that leads to anemia (Agency for Toxic Substances and Disease Registry, 2007). In a recent study by Costa et al. authors assess modifications in circulating levels of advanced oxidation protein products (AOPP), advanced glycation end-products (AGE) and serum reactive oxygen metabolites (ROMs) in a group of gasoline station attendants exposed to low-dose benzene and to evaluate the influence of antioxidant food intake on these biomarkers of oxidative stress and conclude that AOPP are a more sensitive biomarker of oxidative stress in workers exposed to low doses of benzene than AGE (Costa et al., 2016). Chronic exposure to low-dose benzene can modulate signal transduction pathways activated by oxidative stress and involved in cell proliferation and apoptosis. This could represent a possible mechanism of carcinogenic action of chronic benzene exposure (Fenga et al., 2016). Furthermore, it is metabolized into a several intermediate compounds like benzene oxide, catechol, phenol, hydroquinone, and benzoquinones in various organs (Lindstrom et al., 1997). Thus, in addition to benzene, its reactive

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metabolites contribute to the alterations in the functions of enzymes and proteins (Uzma et al., 2010). In 1982, International Agency for Research on Cancer (IARC) declared that there is sufficient evidence that benzene is carcinogenic to man and afterwards classified as a Group 1 carcinogen ("Benzene (IARC Summary & Evaluation, Volume 29, 1982)," n.d.). Minciullo et al. showed that it can either directly damage hematopoietic progenitor cells, leading to the induction of cell death or may alter the responsiveness of these cells to cytokines and cellular adhesion molecules (Minciullo et al., 2014). Additionally, the duration of the interaction might play a role in immune consequences. Chronic exposure to benzene can lead to deleterious effects on many biological systems including both innate and adaptive components of the immune system, on the other hand, it has been shown that low levels of benzene exposure was associated with significant decline in serum IgM and IgA levels (Kirkeleit et al., 2006). Therefore, we aimed to determine whether benzene could modulate lymphocyte proliferation induced by the T cell mitogen Concanavalin A (Con A), in rats, at different exposure durations.

2. Methods

2.1. Animal experiments

The experiments were conducted with 386 healthy male Wistar rats weighing between 250 and 300 g. Rats were allocated into two groups. Group 1 (control) received drinking water only. The rats in Group 2 received in drinking water, 0.6 mL/kg/day benzene at the dose that was equal to one MPC dose (*Hygiene standard 2.1.5.2280-07.2008. "Approximately permissible concentrations (APC) of chemicals in water bodies of drinking, household, cultural-community water use.,"* 2008Hygiene standard, 2008*Hygiene standard 2.1.5.2280-07.2008. "Approximately permissible concentrations (APC) of chemicals in water bodies of drinking, household, cultural-community water use.,"* 2008). At days 45, 90 and 135 of treatments (OECD, 2009, 1998). subsets of rats were euthanized with ether anesthesia. The results of each set of experiments of Group 1 had no difference, thus all the animals at any duration, together constituted the control group. The experiments were conducted in accordance with the ethical standards and recommendations for humanization of work with laboratory animals, covered by the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe, 1985).

2.2. Immunological methods

The number of the cells, thymic index (thymus mass, mg/body mass, g) and splenic index (spleen mass, mg/body mass, g) were defined in blood, thymus and spleen of rats in accordance with the laboratory methods of experimental animal studies (Volchegorskiy et al., 2000).

For the immunophenotyping analysis, rats from each group were randomly selected. Immunophenotyping of splenocytes was conducted using monoclonal antibodies ("eBioscience", USA) against CD3, CD4, CD8 receptors. Percentage of CD3+, CD4+, CD8+ spleen lymphocytes was defined using the flow cytometer "FACS Canto II" with two lasers ("Becton Dickinson", USA).

The splenocytes were cultured at 37 °C, 5% CO_2 in RPMI-1640 medium, supplemented with 10% inactivated fetal calf serum, 2 mM glutamine and 80 μg/mL gentamycine. The cells were further stimulated with 5 μg/mL Con A and at 24 h, interferon-gamma (IFN-γ), IL-4, IL-6, IL-10 were assessed by enzyme linked immunosorbent assay (ELISA,"Bender MedSystems", Austria). The absorbances were measured by using the photometer Multiskan (Labsystems, Finland).

The formation of cellular immunity was studied using the model of local delayed-type reaction. Thymus-dependent antigen response (ram's erythrocytes) was studied by defining antibody producing cells in spleen by Jerne's method and hemagglutinin content in hemagglutination reaction in blood serum (Volchegorskiy et al., 2000). Cell cycle and apoptosis of splenocytes were assessed using DNA fluorochrome staining method, followed by the cytofluorometry using the flow cytometer FACS Calibur (Sibirijak et al., 2008).

2.3. Statistical analysis

The results of the studies were processed using variation statistics methods with the help of software package "Microsoft Excel 7.0", "STATISTICA 10.0", including parametric (the Student's test) and nonparametric analysis methods (the Mann-Whitney *U* test). The results are presented in the form of arithmetic mean value (Mean ± standard error of mean).

3. Results

3.1. Amount of nuclear cells in blood and lymphoid organs

The effect of benzene exposure on leukocytes and total number of lymphocytes were most pronounced at day 90. The number of leukocytes of the Wistar rats were decreased compared to the control group ($6.3 \pm 0.48 \times 10^9$ cell/mL and $10.10 \pm 0.30 \times 10^9$ cell/mL, respectively). Similarly, the absolute number of lymphocytes of the animals were declined in comparison to the untreated group ($5.08 \pm 0.37 \times 10^9$ cell/mL and $8.87 \pm 0.03 \times 10^9$ cell/mL, respectively)

Regarding lymphoid organs, the effect of benzene was expressed by the decrease in thymus mass, spleen mass and number of nuclear cells. The maximum decrease in thymus mass and the nuclear cells number in thymus was observed at Day 135, while the decline was more enhanced in spleen mass at Day 45, and in number of karyocytes in spleen at Day 90 (Table 1). Furthermore, thymic index, number of thymus nuclear cells corresponding to body mass (minimum at Day 135) and corresponding to the organ mass (minimum at Day 45), splenic index, and number of spleen nuclear cells corresponding to the body mass and corresponding to the spleen mass (minimum at Day 90) were decreased.

Thus, the dynamics of changes of nuclear cells number in blood and lymphoid organs of Wistar rats give evidence of the deleterious effects of benzene, which is expressed by leukopenia, decrease in lymphoid organs (thymus and spleen) mass and in the number of lymphoid organs cells. Additionally, thymic and splenic indexes and number of karyocytes in these organs, standardized according to body and organ mass were diminished. These alterations were more pronounced at Days 90 and 135.

3.2. Immunological parameters

Exposure to benzene led to a change in the pattern of T-lymphocyte sub-populations in the spleen of the rats (Table 2). The absolute number of CD3+ cells in the spleen decreased at Days 45, 90, and 135 ($306 \pm 19.16 \times 10^6$ cells, $255 \pm 17.89 \times 10^6$ cells and $362 \pm 24.04 \times 10^6$ cells, respectively) compared to the controls ($452 \pm 36.53 \times 10^6$ cells). Also, the absolute number of CD4+ cells was also decreased to $212.80 \pm 20.02 \times 10^6$cells, $185.23 \pm 8.29 \times 10^6$ cells and $223.00 \pm 22.92 \times 10^6$ cells at these timepoints, respectively, compared to the controls $339.42 \pm 28.01 \times 10^6$ cells. The lowest absolute number of CD8+ cells was noted at Day 90.

The cytokine concentrations after Con A stimulation of the benzene exposed splenocytes are given in Table 3. Compared to the controls, while IL-4 production was significantly increased at all treatment groups, IL-6 levels were decreased and reached to a statistical significance at the Days 45 and 135.

Benzene was found to suppress the cell-mediated and humoral immune responses. It was expressed by the weakening of the delayed-type reaction intensity in the benzene treated rats, 65.71 ± 10.14 mg