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Effect of benzene on the cerebellar structure and behavioral characteristics in rats

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ABSTRACT

Objective: To investigate the effects of benzene on rat's cerebellum structure and behavioral characteristics, including anxiety and motor impairment.

Methods: Twenty rats were randomly allocated into two groups orally receiving distilled water and benzene (200 mg/kg/day). A total of 10 rats were used at the beginning of benzene exposure. Two rats died during benzene treatment and 8 rats remained for evaluation of the behavioral test and finally 6 rats underwent histological assessment. At the end of the 4th week, motor function and anxiety were evaluated in rotarod test and elevated plus maze, respectively. Besides, the cerebellum was dissected for structural assessment using stereological methods.

Results: Performance of the benzene-treated rats in fixed and accelerating speed rotarod was impaired and their riding time (endurance) was lower compared to the control group (P = 0.02). The benzene-treated rats also spent less time in the open arms and had fewer entrances to the open arms in comparison to the control group, indicating anxiety (P = 0.01). The total volume of the cerebellar hemisphere, its cortex, intracerebellar nuclei, total number of the Purkinje, Bergmann, Golgi, granule, neurons and glial cells of the molecular layer, and neurons and glial cells of the intracerebellar nuclei were reduced by 34%–76% in the benzene-treated rats in comparison to the distilled water group (P = 0.003). The most cell loss was seen in Bergmann glia.

Conclusions: The structure of cerebellum altered after benzene treatment. In addition, motor impairment and anxiety could be seen in benzene-treated rats.

1. Introduction

Benzene is an aromatic chemical and can be found in varying amounts in atmosphere, water, soil, and food. Human exposure to benzene in work environment is a universal work-related health problem [1]. Contact with benzene has been related to many different types of blood-related disorders in both animals and humans [2]. Benzene also induces oxidative damage and apoptosis in the nervous system [1,2]. Moreover, benzene has harmful effects on the neurobehavioral functions [3,4]. The results of the study by Lo Pumo R *et al.* indicated that prenatal exposure to benzene could produce long-lasting neurotoxicity [5]. Human contact to benzene can occur not only through breathing and dermal absorption, but also through eating food and drinking water. High concentrations of benzene in the groundwater could cause latent risk to human health and alter the diversity and arrangement of environmental and ecological systems [6]. Natural gas has been also described as a source of benzene contamination in groundwater [6]. Although the effects of benzene on the biochemical, pathological, and physiological aspects of nervous tissue have been explained, the detailed quantitative changes of the cerebellar structure have received less attention. The present study was carried out to find answers to the following questions: how much does the volume of the cerebellum, cortex, medulla, and intracerebellar nuclei alter after treatment of rats with benzene? Does the number of Purkinje, Bergmann, Golgi, granules, neurons, and glial cells of the cortex and the intracerebellar nuclei change after treatment with benzene? Is exposure to benzene associated with impairment in rotarod and elevated plus maze? The rotarod and elevated plus maze tests are used for evaluating the motor coordination and anxiety,

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respectively [7,8]. Unbiased stereological methods were used to estimate the volume and number of the cells of the rats' cerebellum.

2. Materials and methods

A total of 20 Sprague–Dawley adult male rats (170–230 g) were kept under standard conditions [(24 ± 2) °C, 60% air humidity] and had free access to food and tap water. The rats were placed in plastic cages with stainless steel grid covers and wood chips as bedding material under 12 h light/dark cycle. There were three to five rats in per cage. Following an acclimatization period of 8 days, the rats were divided into two benzene-treated and control groups. Two rats died during benzene treatments and 8 rats remained for evaluation of the behavioral test. Finally, 6 rats underwent histological assessment. The use of the animal experiment was approved by the Ethics Committee of the university under approval No. 91-6416. The control and benzene groups received distilled water and benzene (200 mg/kg/day, Sigma–Aldrich, Steinheim, Germany), respectively by oral gavages.

About 2 mL benzene at the dose of 200 mg/kg/day was administered by oral gavages once daily for 4 weeks. The dose of 200 mg was selected according to the previous studies [6,9,10]. Different doses of benzene up to 1000 mg/kg were given orally to the rats in order to evaluate benzene toxicity in the benzene-exposed animals [6,9,10]. Based on the routine toxicity studies, the highest dose, *i.e.*, 800 mg/kg/day, induced toxic effects, but caused no severe suffering, death, or marked growth retardation [6,9,10]. However, no toxic effects were observed at the dose of 10 mg/kg/day. Therefore, dose of 200 mg/kg/day was selected in this study. It should be noted that human contact with benzene can occur through breathing, dermal absorption, eating food, and drinking water [6,9–11]. Therefore, the materials were administered through oral gavages in the present study.

At the end of the treatments, motor impairment and anxiety were assessed by rotarod and elevated plus maze. Then, under deep ketamine/xylazine anesthesia, the cerebellum was removed and the structural study was done on the right cerebellar hemisphere.

2.1. Rotarod performance

The effect of benzene on the rats' motor activities was determined using the rotarod performance test. After initial adaptation, the rats were tested first on fixed speed and two days later on accelerating protocols [8,12]. All the study rats were trained to run on the rotarod at a constant speed (15 r/min) for 60 s on two consecutive days. Then, their latency to fall was assessed. The apparatus could be set on fixed and accelerating speeds. For fixed speed evaluation, the rats were placed on the rod and sequentially tested at 12, 16, 19, 21, 24, 26, 28, and 38 r/min for a maximum of 60 s at each speed. The animals were evaluated three times at each speed with an interval of 20 min between each trial. For accelerating speed evaluation, the rats were placed on the rod and the speed was increased smoothly from 5 to 45 r/min over a period of 300 s. For both evaluations, the duration that each animal was capable to stay on the rod was recorded as the latency to fall [8,12].

2.2. Elevated plus maze

The elevated plus maze apparatus consisted of a central platform (10 cm × 10 cm), two opposite open arms (50 cm × 10 cm × 1 cm height), and two closed arms (50 cm × 10 cm × 40 cm height) [13,14]. The rats were located on the central platform facing an open arm and permitted spending 5 min freely at the maze. The number of entries to open and closed arms with the four paws and the time spent in the arms were recorded. The percentage of open arm entries [open entries/(open + closed entries) × 100] and the percentage of time spent in the open arms [(open time/300) × 100] were calculated, as well. The total number of arm entries and the number of closed-arm entries were usually considered as measures of general activity. It should be noted that the maze was cleaned with a solution of alcohol–water after each trial [13,14].

2.3. Estimation of the total volume

The cerebellum was fixed in neutral buffered formaldehyde for one week and after processing, it was stained using cresyl violet. Coronal sections (26 μm thickness) from the right hemisphere were prepared and stained with cresyl violet. Overall, 10 to 12 sections were sampled through systematic uniform random sampling to estimate the total volume of the cerebellar hemisphere and cortex. Another set of 10–12 sections were also sampled in order to estimate the total volume of the intracerebellar nuclei. The lateral, interposed, and medial nuclei were considered collectively. Using a projecting microscope and Cavalieri's principle, the volume was estimated at the final magnification of 25× [15,16]. Briefly, the distances between the sampled sections were calculated. Besides, the area was evaluated using point counting method. Accordingly, the area per point was 0.84 mm^2 and averagely 150–250 points were counted per animal. Finally, the volume was evaluated using the following formula:

$$V = (a/p) \times \sum P \times d$$

Where, a/p was the area per point; $\sum P$ was averagely 150–250 points and d was the distances between the sampled sections.

2.4. Estimation of the total cell number

A computer linked to a light microscope (Nikon E200, Nikon, Japan) and an oil immersion lens (60×, numerical aperture: 1.4) were used in order to estimate the total number of the cells of the cerebellum by the optical disector method. The microscopic fields were sampled by moving the microscope stage in an equivalent interval using a stage micrometer [15–19]. Using a microcator (MT12, Heidenhain, Germany) connected on the stage, the z-axis movement of the microscope stage was measured [15–19]. In summary, an unbiased counting frame with area of 846 μm^2 with acceptance (right and upper) and forbidden (left and lower) borders was superimposed on the images of the tissue sections viewed on the monitor. To obtain the appropriate guard zone and the height of the disector, z-axis distribution of nuclei was plotted [17]. Briefly, the counted cells were scored and grouped in 10 columns from percentiles 0–100 through the histological tissue section from the upper (0%) to the lower surface (100%) (Figure 1). The upper and lower 30% of the histogram were discarded as the guard zones and the counting box was located on the remaining 40% (h). According to the histogram, the counting was corrected [15–19]. Any cell nucleus which came into the focus within the sampling box ($h \times a/f$)