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

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2. Materials and methods

A total of 20 Sprague-Dawley adult male rats (170-230 g) were kept under standard conditions [(24 ± 2) ℃, 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 1 000 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 seconds 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 seconds 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 seconds. 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 to 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.

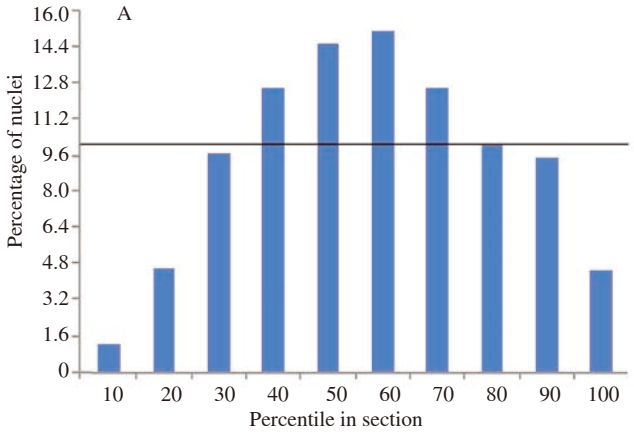
2.4. Estimation of the total cell number

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 ($h \times a/f$) was selected if it was located completely or partly inside the counting frame and did not touch the forbidden borders (Figure 1). The total number of the cells was estimated by multiplying the numerical density by the total volume of the cortex of intracerebellar nuclei:

$$Nv\ (neurons/nuclei) = \frac{\sum Q^-}{\sum P \times \frac{a}{f} \times h} \times \frac{t}{BA}$$

Where, Nv was numerical density; ΣQ^- was the number of the nuclei coming into focus during scanning (Figure 1); ΣP was the total number of unbiased counting frames in all fields; h was the

height of the disector; *a/f* was the frame area; *t* was the section thickness measured in every sampled field using the microcator (23 μm on the average), and *BA* was the block advance of the microtome which was set at 26 μm[15-19].



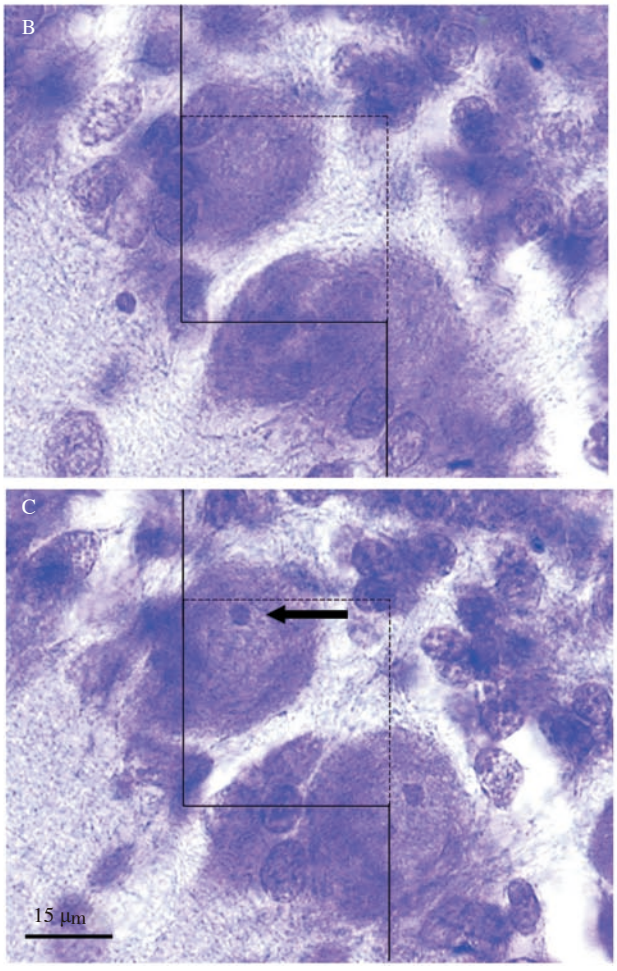


Figure 1. Cell counting method using optical disector.
A: Z-axis distribution of the cerebellar cells; B and C: The optical sections used to estimate the cells in the counting frame (the arrow).

2.5. Estimation of the coefficient of error (CE)

CE (V) was calculated by the following formula:

$$CE(V) = (\sum P)^{-1} \times [1 / 240 (3 \times \sum P_i P_i + \sum P_i P_{i+2} - 4\sum P_i P_{i+1}) + 0.0724 \times b / (a^{1/2}) \times (n \sum P_i)^{1/2}]^{1/2}$$

Where, b and a indicated the mean section boundary length and mean sectional area, respectively[15-19].

2.6. Statistical study

The animals' performance in rotarod and elevated plus maze were analyzed using repeated measures and Two-way ANOVA, respectively. Besides, Mann-Whitney *U*-test was used to analyze the histological data. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Rotarod performance

The performance of the benzene-treated rats in the fixed speed test was weakened and the riding time was 42 seconds at the lowest speed and 24 seconds at the highest speed in comparison to the distilled water group (60-49 seconds). Additionally, the performance of the benzene-treated rats in the accelerating speed test was abated and their riding time was 72% lower compared to the distilled water group (Figure 2).

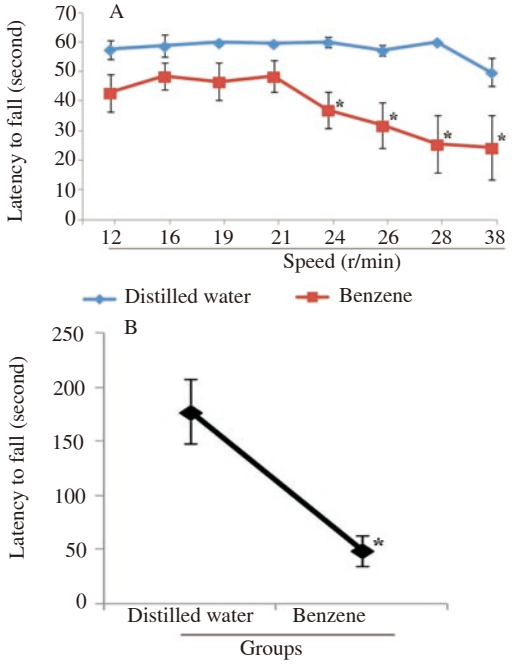


Figure 2. The plot showing the mean and SE bars of the latency to fall (s) of rats in rotarod tests in the groups treated with distilled water and benzene.
A: Fixed speed test (speeds have been shown as r/min); B: Accelerating speed test; *: $P = 0.01$.

3.2. Elevated plus maze

The time spent in the open arms by the benzene-treated rats was 66% lower than that of the control group ($P = 0.01$). In addition, their number of open arm entries was 56% fewer in comparison to the distilled water group. The general motor activity of the benzene-treated rats was also 31% less than that of the control rats ($P = 0.01$) (Figure 3).

3.3. Stereological study

CE of the total volume estimation in both distilled water and benzene-treated groups was 0.03 ± 0.01.

The total volume of the cerebellar hemisphere, its cortex, and medulla was reduced by 34% in the benzene-treated rats in comparison to the distilled water group ($P = 0.003$) (Figure 4).

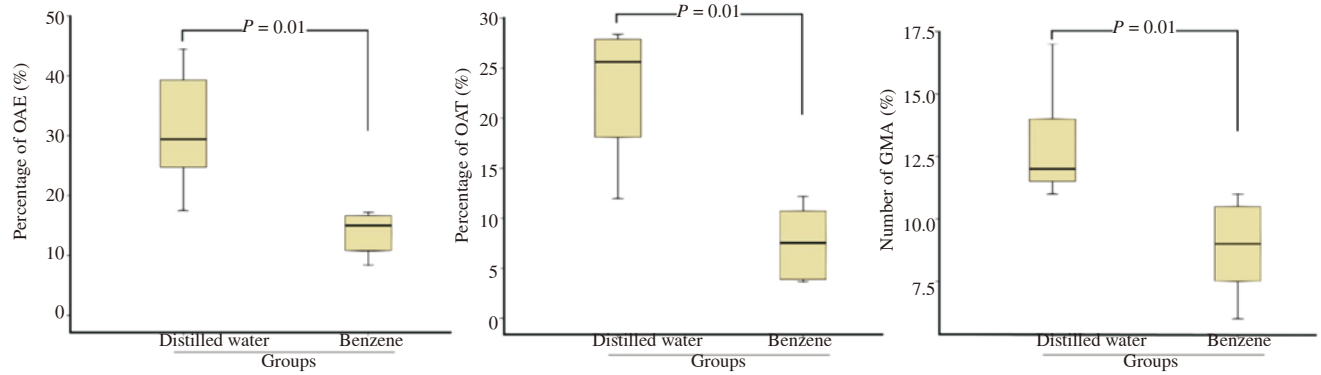


Figure 3. Box-plot diagram showing the rats' performance in elevated plus maze in the groups treated with distilled water and benzene.
The box shows median value and the 25th and 75th percentiles; the whiskers show the min and max values. OAE: open arm entrances; OAT: Percentage of open arm time; GMA: Number of general motor activities; The differences ($P = 0.01$) have been indicated.

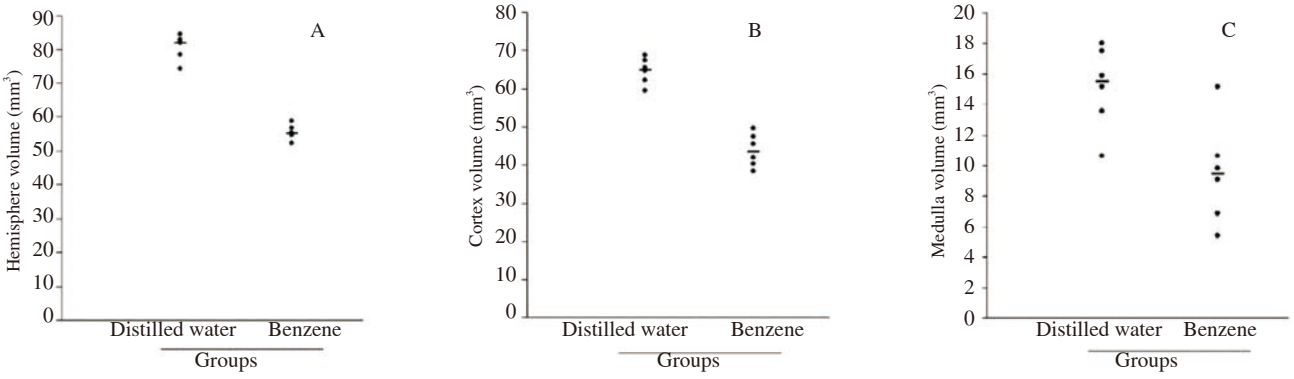


Figure 4. The scatter plots of the volume (mm^3) of the cerebellar hemisphere, its cortex and medulla.
Each dot represents an animal and the horizontal bar is the median value of the parameter in each group.

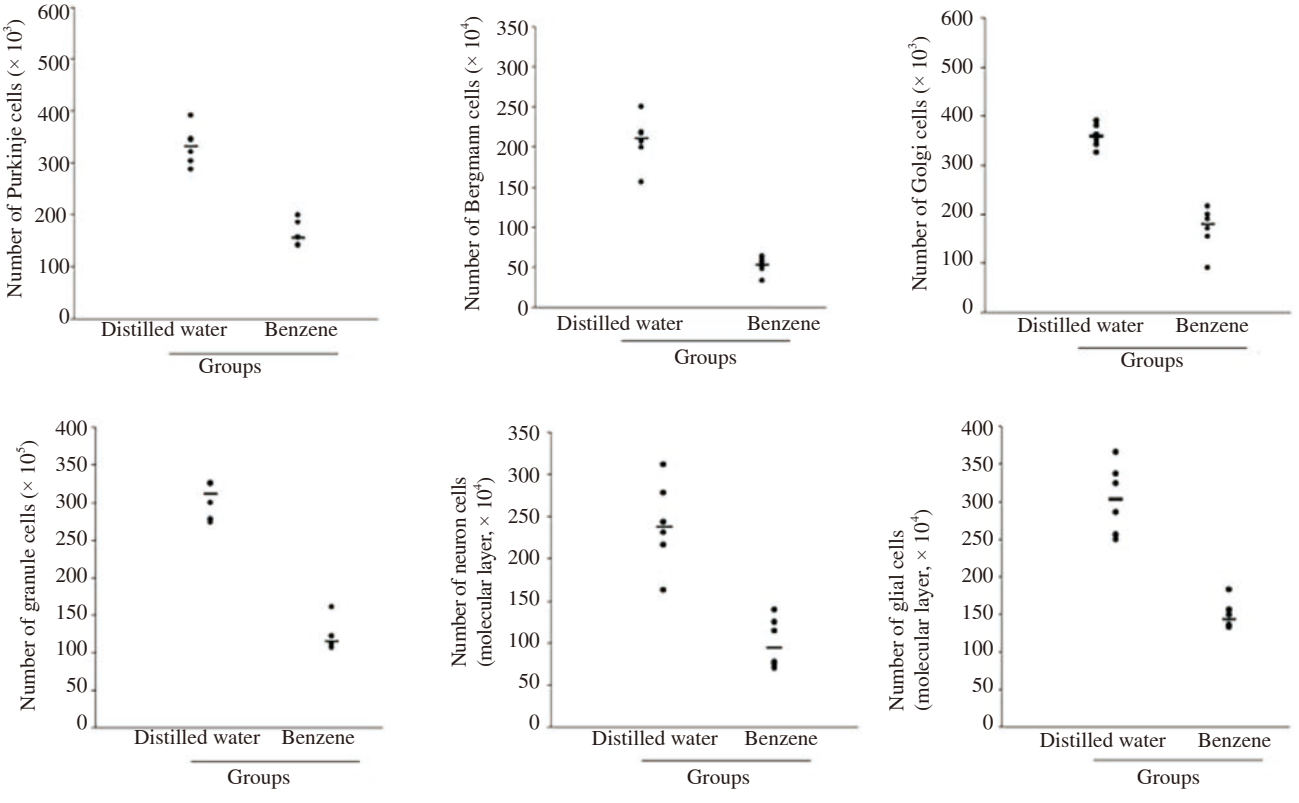


Figure 5. The scatter plots of the number of the cortical cerebellar cells.
Each dot represents an animal and the horizontal bar is the median value of each group.

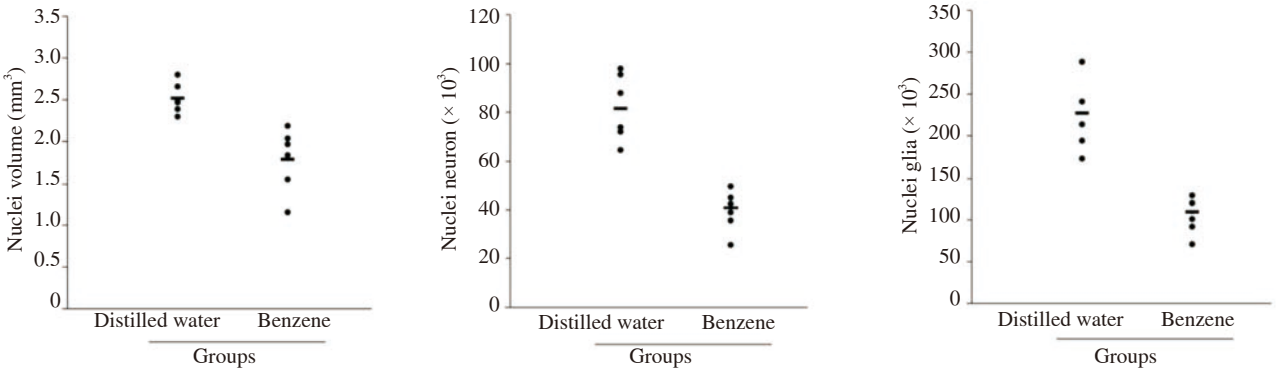


Figure 6. The scatter plots of the volume (mm^3) and number of the cells of intracerebellar nuclei. Each dot represents an animal and the horizontal bar is the median value of each group.

Moreover, the total number of the Purkinje, Bergmann, Golgi, granule cells, neurons, and glial cells in molecular layer of the benzene-treated rats was respectively 52%, 76%, 51%, 62%, 58%, and 50% lesser than that of the distilled water group (P = 0.003) (Figure 5). The maximum cell loss could be seen in Bergmann cell.

The total volume of the intracerebellar nuclei of the hemisphere was reduced by 28% in the rats treated with benzene in comparison to the distilled water-treated group (P = 0.003) (Figure 6). Additionally, the total number of the neurons and glial cells of the intracerebellar nuclei respectively decreased by 51% and 54% in the rats treated with benzene in comparison to the distilled water-treated group (P = 0.003) (Figure 6).

3.4. Histological evaluation

Qualitative histological evaluation of the cerebellar hemispheres also revealed changes in the benzene-treated animals (Figure 7). Evaluation of the cortex and nuclei showed fewer cells in these areas.

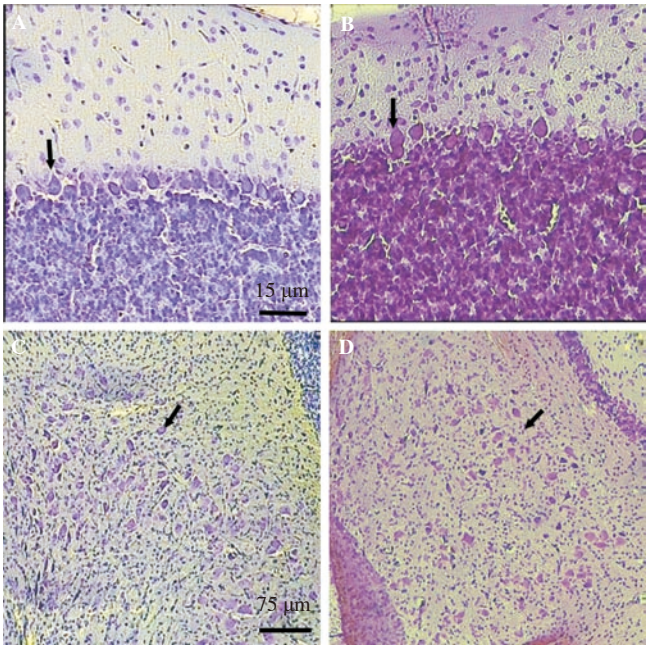


Figure 7. Photomicrograph of the cerebellar cortex and nuclei stained with cresyl violet.
The population of the cells in the cortex decreased in the benzene-treated rats (B) in comparison to the distilled water group (A). Fewer Purkinje cells (arrow) were recognized after exposure to benzene. The population of the cells (arrow), including neurons and glial cells of the intracerebellar nuclei decreased after treatment with benzene (D) compared to distilled water (C).

4. Discussion

The present study analyzed alteration in motor activity and quantitative histological changes of the rats' cerebellum after exposure to benzene.

In this study, benzene was administered through oral gavages. Although little amounts of benzene have been detected in certain foods, drinks and tap water, these do not constitute main sources of contact for most individuals. However, outflow from subversive gasoline storage reservoirs and oozing from landfills and harmful waste sites have resulted in noteworthy benzene contamination of well water[6].

It has been reported that environmental toxins including benzene derivatives may cause cerebellar deficits[20-22]. Acute exposure to benzene in pregnant rats may induce progeny long-lasting behavioral changes; *i.e.*, reduced motor activity and cognitive capacity[5]. The results of the previous studies indicated that benzene also caused neurobehavioral alteration in petrochemical workers[20]. Evaluation of the neurobehavioral effects of benzene by Chalansonnet *et al*. also showed that this compound induced a temporary depressant effect on the rats' central nervous system. These effects included alterations in posture, reduction of arousal and rearing, simplicity of handling, instability of gait, mobility, righting reflex, reduced forelimb grip strength and impaired motor coordination[21]. The above-mentioned studies are all in agreement with our study. Manto explained that the cerebellar cortex and Purkinje neurons were the main targets of environmental toxins, including benzene derivatives[22]. That study emphasized that benzene was one of the compounds that could induce cerebellar ataxias, which is consistent with our structural findings[22]. However, the results of the current study showed that not only the cortical cells but also the cells of the intracerebellar nuclei were affected by benzene. More than 50% of all cerebellar cells were lost after benzene poisoning in the rats. The most cell loss was related to Bergmann cells. Therefore, the loss of the cerebellar cells can explain the motor deficit after benzene exposure. Bergmann cells are the astrocytes in the cerebellum that have their perikarion in the Purkinje cell layer and their processes spread into the molecular layer[23]. Bergmann cells limit dispersal of the neurotransmitters and are also essential for trimming or addition of synapses[23]. It has been reported that disruption of the architecture of Bergmann glia led to neurodegeneration of cerebellar Purkinje cells[23]. Thus, the extensive loss of the supporting Bergmann cells in the current study might explain a reason for the loss of Purkinje cells.

Our study findings showed a massive glia cells loss in the benzene-treated animals. These cells are responsible for maintaining homeostasis, myelin formation and providing support and protection for neurons in the nervous system, including cerebellum[24]. In addition, research indicated that glial cells of the cerebellum participated in synaptic transmission; regulated the clearance of neurotransmitters from the synaptic cleft and released gliotransmitters (such as ATP) which modulated synaptic function[24]. Hence, the glial cells loss induced by benzene could be followed by neuron loss and dysfunction which was appeared as deficit in motor performance in rotarod.

The limitation of the current study was absence of immunohistochemical evaluation and quantification of different kinds of cell death, including apoptosis, necrosis and autophagy. The results of the current study can be tracked by future researches for identifying the types of cell death and the related mechanisms after benzene consumption.

The findings of the present study revealed that treatment of the rats with 200 mg/kg/day benzene for 4 weeks could induce anxiety, indicated by elevated plus maze. The riding times in fixed and accelerating speed rotarod also reduced, indicating motor mutilation. The cerebellar structure was also altered after the treatment. Similarly, the volume of the hemisphere, cortex and intracerebellar nuclei and their number of cells reduced in the benzene-treated rats.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

[1] Snyder R. Benzene's toxicity: a consolidated short review of human and animal studies by HA Khan. *Hum Exp Toxicol* 2007; **26**: 687-96.

[2] Turteltaub KW, Mani C. Benzene metabolism in rodents at doses relevant to human exposure from urban air. *Res Rep Health Eff Inst* 2003; (113): 1-35.

[3] Manno M. [Update on benzene: from industrial toxicant to environmental carcinogen]. *G Ital Med Lav Ergon* 2013; **35**: 247-50. Italian.

[4] Wang F, Li C, Liu W, Jin Y. Potential mechanisms of neurobehavioral disturbances in mice caused by sub-chronic exposure to low-dose VOCs. *Inhal Toxicol* 2014; **26**(4): 250-8.

[5] Lo Pumo R, Bellia M, Nicosia A, Micale V, Drago F. Long-lasting neurotoxicity of prenatal benzene acute exposure in rats. *Toxicology* 2006; **223**: 227-34.

[6] Wilbur S, Wohlers D, Paikoff S, Keith LS, Faroon O. ATSDR evaluation of potential for human exposure to benzene. *Toxicol Ind Health* 2008; **24**: 399-442.

[7] Hazim AI, Ramanathan S, Parthasarathy S, Muzaimi M, Mansor SM. Anxiolytic-like effects of mitragynine in the open-field and elevated plus-maze tests in rats. *J Physiol Sci* 2014; **64**: 161-9.

[8] Noorafshan A, Erfanizadeh M, Karbalay-Doust S. Sodium benzoate, a food preservative, induces anxiety and motor impairment in rats. *Neurosciences (Riyadh)* 2014; **19**: 24-8.

[9] Heijne WH, Jonker D, Stierum RH, van Ommen B, Groten JP. Toxicogenomic analysis of gene expression changes in rat liver after a 28-day oral benzene exposure. *Mutat Res* 2005; **575**: 85-101.

[10] Hasegawa R, Hirata-Koizumi M, Takahashi M, Kamata E, Ema M. Comparative susceptibility of newborn and young rats to six industrial chemicals. *Congenit Anom (Kyoto)* 2005; **45**: 137-45.

[11] Minciullo PL, Navarra M, Calapai G, Gangemi S. Cytokine network involvement in subjects exposed to benzene. *J Immunol Res* 2014; doi: 10.1155/2014/937987.

[12] Shiotsuki H, Yoshimi K, Shimo Y, Funayama M, Takamatsu Y, Ikeda K, et al. A rotarod test for evaluation of motor skill learning. *J Neurosci Methods* 2010; **189**: 180-5.

[13] Sestakova N, Puzserova A, Kluknavsky M, Bernatova I. Determination of motor activity and anxiety-related behaviour in rodents: methodological aspects and role of nitric oxide. *Interdiscip Toxicol* 2013; **6**(3): 126-35.

[14] van Dijk A, Klanker M, van Oorschot N, Post R, Hamelink R, Feenstra MG, et al. Deep brain stimulation affects conditioned and unconditioned anxiety in different brain areas. *Transl Psychiatry* 2013; **3**: e289.

[15] Altunkaynak BZ, Onger ME, Altunkaynak ME, Ayranci E, Canan S. A brief introduction to stereology and sampling strategies: basic concepts of stereology. *Neuroquantology* 2012; **10**: 31-43.

[16] Kristiansen SL, Nyengaard JR. Digital stereology in neuropathology. *APMIS* 2012; **120**: 327-40.

[17] von Bartheld CS. Distribution of particles in the z-axis of tissue sections: relevance for counting methods. *Neuroquantology* 2012; **10**: 66-75.

[18] Geuna S, Herrera-Rincon C. Update on stereology for light microscopy. *Cell Tissue Res* 2015; **360**(1): 5-12.

[19] Rafati A, Anvari E, Noorafshan A. High fructose solution induces neuronal loss in the nucleus of the solitary tract of rats. *Folia Neuropathol* 2013; **51**: 214-21.

[20] Zhang M, Wang Y, Wang Q, Yang D, Zhang J, Wang F, et al. Ethylbenzene-induced hearing loss, neurobehavioral function, and neurotransmitter alterations in petrochemical workers. *J Occup Environ Med* 2013; **55**: 1001-6.

[21] Chalansonnet M, Carabin N, Boucard S, Cosnier F, Nunge H, Gagnaire F. Study of the potential oxidative stress induced by six solvents in the rat brain. *Neurotoxicology* 2013; **35**: 71-83.

[22] Manto M. Toxic agents causing cerebellar ataxias. *Handb Clin Neurol* 2012; **103**: 201-13.

[23] Wang X, Imura T, Sofroniew MV, Fushiki S. Loss of adenomatous polyposis coli in Bergmann glia disrupts their unique architecture and leads to cell nonautonomous neurodegeneration of cerebellar Purkinje neurons. *Glia* 2011; **59**: 857-68.

[24] Jebelli J, Su W, Hopkins S, Pocock J, Garden GA. Glia: guardians, gluttons, or guides for the maintenance of neuronal connectivity? *Ann N Y Acad Sci* 2015; doi: 10.1111/nyas.12711.