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Experimental Neurobiology

Original Article

Curcumin Can Prevent the Changes in Cerebellar Structure and Function Induced by Sodium Metabisulfite in Rat

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Sulfites are used as anti-microbial and anti-oxidant agents in the food and pharmaceutical industries. Curcumin, a flavonoid, is an Asian spice that shows neuroprotective activities. The current study aimed to stereologically assess the rats' cerebellar cortex and rotarod performance following sulfite exposure and determine the possible neuroprotective potential of curcumin. The rats were divided into five groups: distilled water, olive oil, curcumin (100 mg/kg/day), sodium metabisulfite (25 mg/kg/day), and sodium metabisulfite+curcumin. At 56 days after treatment, rotarod performance was tested, and then the cerebellum was removed for stereological analysis. The study results revealed 31%, 36%, 19% and 24% decrease in the total volume of the cerebellum, cortex, the total number of the Purkinje cells and length of the nerve fibers in the cortex per Purkinje, respectively in the sodium metabisulfite-treated rats compared to the distilled water group (p<0.01). The pre-trained animals on the rotarod apparatus were tested first on the fixed speed rotarod protocol followed by the accelerating rotarod protocol two days later. The results showed a significant decrease in the latency to fall in both test in sulfite-treated rats. The sulfite effects on the structural parameters and rotarod performance were significantly protected by the concomitant curcumin treatment (p<0.001). Sulfite can induce structural and functional changes in the rats' cerebellum and concomitant curcumin prescription plays a neuroprotective role.

Key words: cerebellum, sulfite, curcumin, rat, histology

INTRODUCTION

Sulfiting agents are food preservatives composed of potassium or sodium bisulfite or potassium metabisulfite. Sulfiting agents are used in the processing of some drinking, baked goods, soup mixes, and some imported sea foods and by restaurants to ration a "fresh" appearance to salad fruits and vegetables. These compounds act as antimicrobials and antioxidants [1]. Sulfite can cause cellular toxicity by reacting with a variety of humoral and cellular components [2]. Catabolism of amino acids and other compounds that contain sulfite can naturally generate large quantities of sulfite in the body [2].

However, sulfite oxidase is an enzyme (located in the mitochondria) which oxidizes sulfite to sulfate. This protects the cells from the toxic effects of sulfite [3]. If sulfite oxidase is deficient as in a hereditary disorder, this detoxification process cannot occur. Finally, this leads to neurological abnormalities, such as decrease

in brain growth and mental retardation [3]. Activity of the enzyme is low in the brain, spleen, and testis [3]. Therefore, the chance of sulfite toxicity of neurons is high. It has been shown that sulfite can induce toxic effects on neurons. For example, exposure to sulfite reduced the hippocampal neuron number in rats [4]. In addition, sulfite treatment increased the excitability of the spinal reflexes [5]. It has also been reported in an in vitro study that sulfite and peroxynitrite anion induce toxic effects on neuronal cell lines [6].

Therefore, it is necessary to design a study in order to evaluate the effects of sodium bisulfite on different functional activities of different brain regions. Therefore, at the first step of the present study, the cerebellar cortex structure and the rotarod performance is tested after treatment of sulfite in an animal model. The second step of the study involves introducing a compound that could be added to the foods and be able to protect the motor coordination after sulfite consumption. Curcumin is a yellow Indian spice that has useful pharmacological effects, such as antioxidant, anti-inflammatory, antiapoptotic, anticancer, and anti-infectious effects [7]. Up to now, many neuroprotective effects of curcumin have been shown. In addition, our previous studies have shown that curcumin displays protective effects on the sciatic nerve and dorsal root ganglion following crush in rats [8]. It has also been shown that curcumin has protective effects against neural disorders including diabetic neuropathy and cerebral ischemia [7]. The rotarod test is an instrumental technique for assessment of motor function in experimental animals that was described to evaluate lesion and drug effects on the motor coordination of the rodents due to damage of the basal ganglia and cerebellum [9]. In the present study, the rats were tested in two different protocols, including incremental fixed speed and accelerating speed after prescription of sulfite and curcumin [9]. The present research tries to answer the following questions:

How much does the cerebellar, cortical, and white matter volume change after sulfite-treatment in rats? Will the number of Purkinje cells change after sulfite-treatment in rats? Will the nerve fiber length change after sulfite-treatment in rats? Will the rotarod performance is impaired after sulfite treatment? Can curcumin prevent the possible changes after sulfite-treatment in rats?

MATERIALS AND METHODS

Animals and treatment

Fifty Sprague-Dawley male rats (weight, 250 to 280 g) were sampled from the laboratory animal center of Shiraz University of Medical Sciences, Shiraz, Iran. All the procedures were performed under the supervision of the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran and the animals

were kept under standard condition. The ethics committee of the university agreed with the animal experiment under approval No 91-6070. The animals were randomly divided into five groups each including ten rats. Six rats in each group underwent the stereological study but all ten rats evaluated in rotarod test. The first and the second groups received distilled water and olive oil, respectively as the vehicles for sodium metabisulfite and curcumin. Besides, the third and fourth groups received sodium metabisulfite (25 mg/kg/day) and curcumin (100 mg/kg/day), respectively [4, 8, 10]. Finally, the fifth group received both sodium metabisulfite and curcumin in doses described above. All the administrations were done orally for 56 days. According to the studies of Akdogan et al. [4] and Ribera et al. [10] the time point was adopted. The study of Ribera et al. 2001 was performed on the rats for 28 (subacute) and 85 (subchronic) days of dietary contact [4, 10]. The time point adopted here (56 days) was selected as the average time point which was considered for subacute and subchronic toxicities. The animals were housed in plastic cages under a 12 h/12 h Light/Dark cycle with room temperature of 22±2°C.

Estimation of the volumes of cerebellum, cortex, and white mater

At the end of the 56th day, all the animals were sacrificed and their brains were dissected immediately. Then, the right cerebellar hemisphere was separated. The selected hemispheres were processed and serial coronal sections of 26-mm thickness were obtained and stained with cresyl violet. Cresyl-violet is used to demonstrate the nissl substance in the neurons and cell nuclei. It can be seen from Figure 1 that the cortex and white matter are distinguishable. Overall, 10 to 12 sections were sampled through systematic uniform random sampling. To locate the cerebellum regions, the rat brain atlas by Paxinos and Watson [11] was applied. Using a projecting microscope, the total volumes of the cerebellum, cortex, and white matter were estimated at the final magnification of 25× using Cavalieri's principle [12-14] (Fig. 1A, B). In doing so, the product of the areas and the distances between the sampled sections (d) were calculated. The area was estimated using point-counting method. The area per point (a/p) was 0.36 mm^2 and on the average, 500 points were counted per animal. Finally, the volume was estimated using the following formula:

$$V \text{ (cerebellum)} = (a/p) \times \Sigma P \text{ (cerebellum)} \times d$$

Estimation of the total number of Purkinje cells

A computer connected to a Nikon E200 microscope (Nikon, Japan), ×60 oil immersion objective lens with a high numerical aperture (NA: 1.4), and an optical disector were used in order to
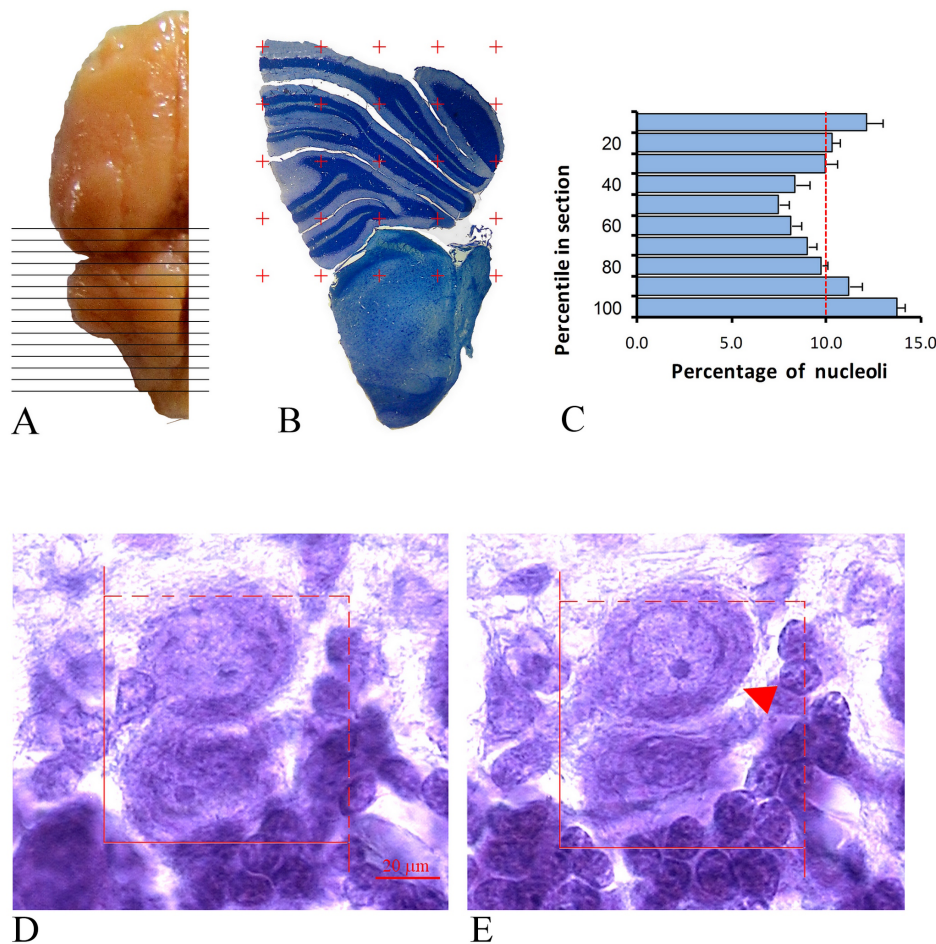
A   B   C

D   E

Fig. 1. Volume and number study. (A) An example of sectioning used to estimate the volume of the cerebellum. The cerebellum was sectioned in coronal plane and the favored sections were sampled with equal distances and random start. (B) Point-counting methods. (C) Z-axis distribution of the Purkinje cell nuclei. The counted Purkinje were organized in 10 histograms from the percentiles 0-100 through the tissue section. The vertical dashed line indicates how the particles would present if all the visible nuclei were equally distributed throughout the Z–axis. (D. E) An unbiased counting frame was superimposed on the micrographs of the cerebellum. Any cell whose nuclei appeared during scanning of the height of the disector and lay on the frame or its accepted borders (dotted line) was counted (arrow).

estimate the total number of the Purkinje cells. The microscopic fields were sampled by moving the microscope stage in an equal interval using a stage micrometer and systematic uniform random sampling [12-14]. The movement of the microscope stage in the Z-axis was measured using a microcator (MT12, Heidenhain, Germany) attached to the stage [15]. Briefly, an unbiased counting frame with inclusion (right and upper) and exclusion (left and lower) borders was superimposed on the images of the sections viewed on the monitor. To calculate the suitable guard zone and the height of the disector (h), Z-axis distribution of the nuclei was plotted. The counted neurons were scored and grouped in 10 bins from 0-100 percentiles through the brain tissue section from the upper surface (0%) to the lower surface (100%). Fig. 1C shows the Z-axis distribution of the Purkinje cell nuclei. The upper and lower 10% of the histogram were considered as the guard zones and the counting box was placed at the remaining 80% (h). According to the histogram, the under-sampling was balanced out and corrected. Any nucleus coming into the maximal focus within the focal sampling plane was selected if it was located completely or partly inside the counting frame and did not touch the exclusion line (Fig. 1D, E).

The total number of the Purkinje cells was estimated by multiplying the numerical density (Nv) by the total volume of the cerebellar hemisphere.

$$\text{Nv (Purkinje/cerebellum)} = \frac{\Sigma Q^-}{\Sigma p \times \frac{a}{f} \times h} \times \frac{t}{BA}$$

where "ΣQ-" was the number of the nuclei coming into focus during scanning the section thickness. On the average, 120~240 Purkinje cells were counted per cerebellum. "ΣP" was the total counting of the unbiased counting frame in all fields, "h" was the height of the disector, "a/f" was the frame area (here was 2,500 μm^2), "t" was the real section thickness measured in every field using the microcator (20 µm), and BA was the block advance of the microtome which was set at 26 µm. The total number of the neurons was estimated by multiplying the numerical density (Nv) by the V (cerebellum).

Estimation of the coefficient of error (CE)

The CE for the estimate of the volume using point counting is the function of the noise effect and systematic random sampling variance for the sums of the estimated areas. Since the cross-sectional areas of the region of interest were estimated by point counting, CE (V) was calculated by the following formula [16-18]:

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

Where "*b*" and "*a*" represented the mean section boundary length and mean sectional area, respectively. The CE for the estimate of the total Purkinje cell number, CE(N), was calculated using CE(V) and CE(Nv) as follows:

$$CE(N) = [(CE^2(Nv) + CE^2(V)]^{1/2}$$
$$CE(Nv) = [(n/n-1) \times [(\Sigma(Q^-)^2 / \Sigma Q \text{-} \Sigma Q^-) + (\Sigma(P)^2 / \Sigma P\Sigma P) - (2\Sigma(Q^- P)/\Sigma Q^-\Sigma P)]]^{1/2}$$

Table 1. Coefficients of error (CE) for the total volume of the cerebellum, number as well as numerical density of the Purkinje cell in the cerebellum

Groups	Volume	Numerical density	Total number
Distilled water	0.05	0.04	0.05
Olive oil	0.03	0.05	0.06
Curcumin	0.04	0.06	0.06
Sulfite	0.03	0.04	0.04
Sulfite+curcumin	0.05	0.06	0.07

The data are shown in Table 1.

Estimation of the nerve fibers length

Vertical uniform random sectioning is necessary for length estimation [18, 19]. Briefly, 9~10 cylinders were punched out using a trocar with diameter of 1 mm vertical to the pial surface of the cortex of the cerebellum (Fig. 2A). The cylinders were randomly rotated along their vertical axis and embedded in one paraffin block. Then, 100 µm thickness slabs were obtained using a microtome and mounted on the slide (Fig. 2B). The dendritic tree of the purkinje cells, parallel fibers of the granular cells, process of the basket, and stellate cells are present in the molecular layer of the cortex. The mean length of the nerve fibers per purkinje was calculated using the following formula [18, 19]:

I_N= (nerve fiber length in the population) / (Purkinje in the population).

A fixed slab height of T (here 100 µm) was scanned inside the slice thickness using a microscope (Nikon E-200) equipped with an objective lens 100× and numerical aperture of 1.4 connected to a computer. Then, the number (Q^-) of the cell bodies of the purkinje cells was computed using the optical disector method. A cycloid was superimposed on the images of the nerve fibers (Fig. 2C). The total number of the intersections (*I*) between axes of the nerve fibers and the cycloid oriented parallel to the vertical axis was calculated, as well (Fig. 2D, E).

$$\bar{I}_N = 2.\frac{a}{l}.\frac{1}{asf}.M^{-1}.\frac{\Sigma I}{\Sigma Q^-}$$
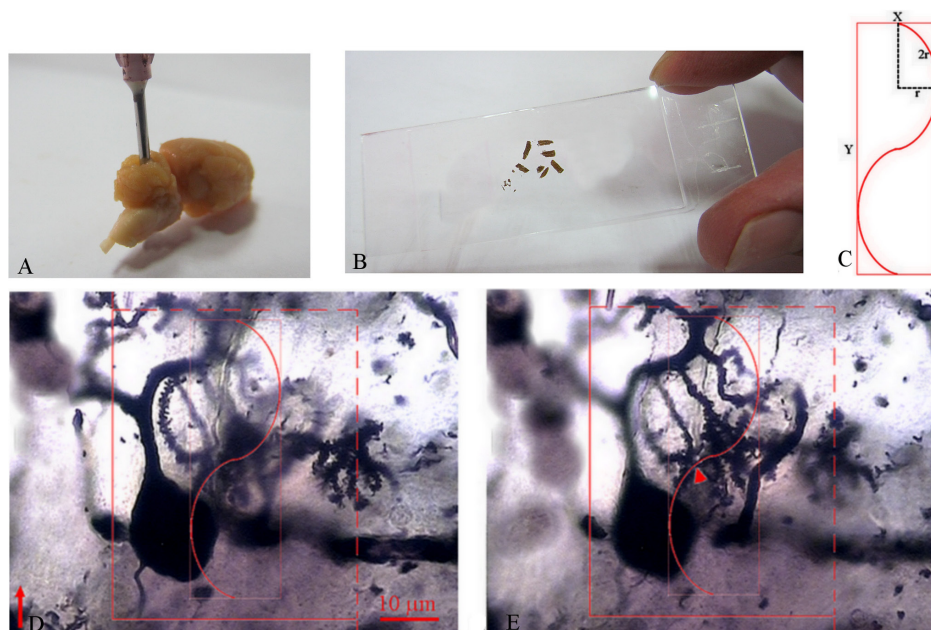

A

B

C

D

E

Fig. 2. Estimation of the length of the nerve fibers. (A) Vertical sectioning. The vertical cylinders were punched out from the cortex of the cerebellum perpendicular to its pial surface. (B) The cylinders were randomly rotated along their vertical axis, sectioned, and mounted on a microscopic slide. (C) Four cycloids were located at a rectangle. The length of each cycloid was equal to twice the length of the minor axis (r). The area associated with the cycloids was calculated by multiplying the X by Y. (D, E) When the sections were scanned, the number of the cell bodies of the neurons was counted using the optical disector method and unbiased counting frame. The total number of the intersections between the nerve fiber axes and the cycloid were counted (arrow head). The cycloid was positioned parallel to the vertical axis (arrow).

Where $\frac{a}{1}$ was the test area per cycloid test length, "asf" was the area associated with cycloid grid divided by the area of the counting frame, and "M" was the final magnification (here 4000) [18, 19].

Rotarod test

Briefly, we pre-trained the animals on the rotarod apparatus. In this study, after initial habituation, the rats were tested first on the fixed speed rotarod protocol and, two days later, on the accelerating rotarod protocol [9]. On the testing day, according to the fixed
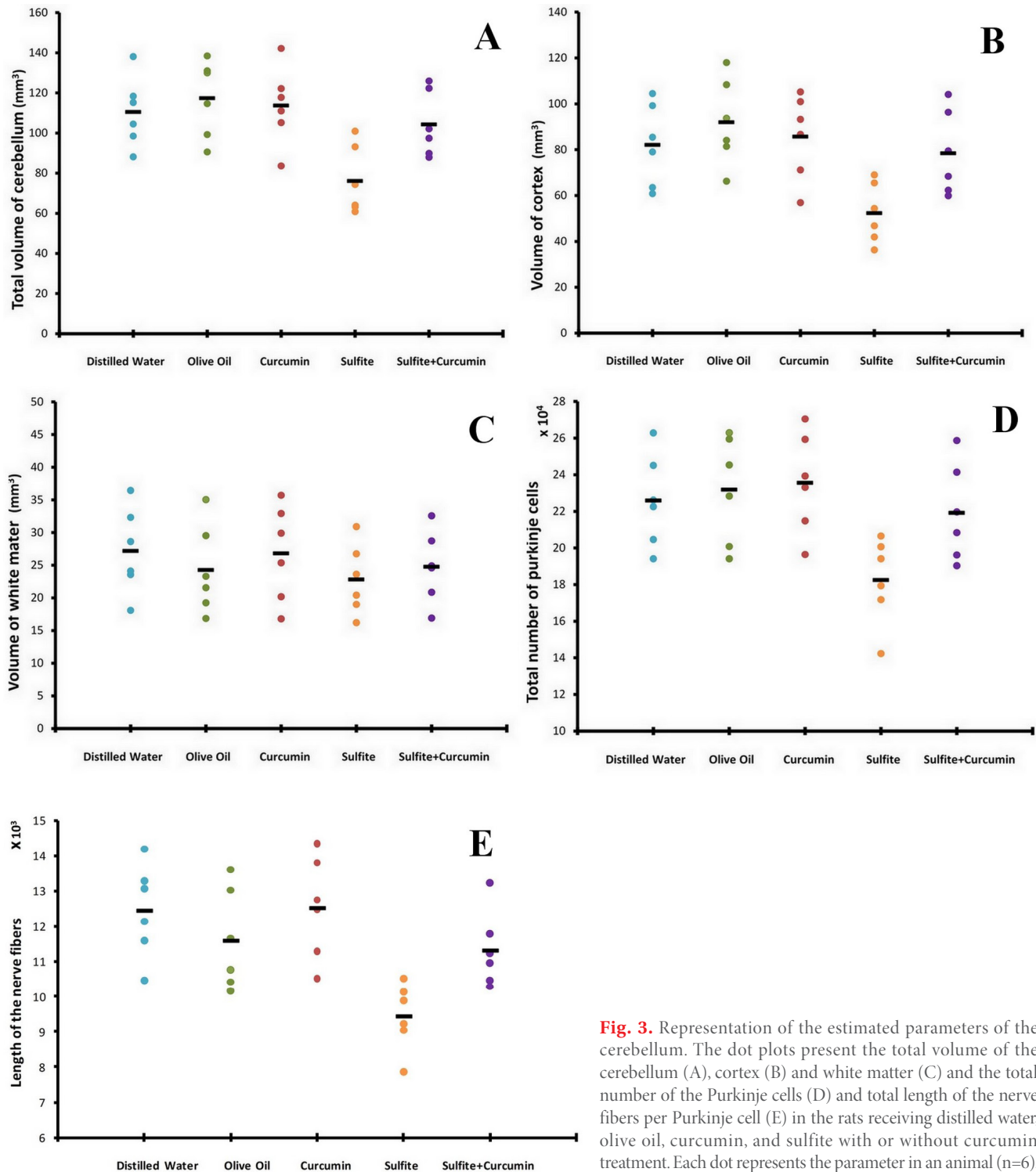

Fig. 3. Representation of the estimated parameters of the cerebellum. The dot plots present the total volume of the cerebellum (A), cortex (B) and white matter (C) and the total number of the Purkinje cells (D) and total length of the nerve fibers per Purkinje cell (E) in the rats receiving distilled water, olive oil, curcumin, and sulfite with or without curcumin treatment. Each dot represents the parameter in an animal (n=6).

speed protocol, the animals were tested at 7 different speeds, including 12, 16, 19, 21, 24, 26, 28, and 38 rpm for a maximum of 60 s at each speed. The rats were tested for three times at each speed with an interval of 20 min between each trial. Latency of each rat to fall from the rotarod was registered automatically with stop watches connected to the detectors [17]. According to the accelerating protocol, the animals were placed on the rod whose rotation speed steadily increased from 4 to 40 rpm during a 300 s period. The latency to fall was recorded as described previously [9].

Statistical analysis

The stereological data were analyzed using Kruskall-Wallis and Mann–Whitney's U-tests. Besides, p<0.05 was considered as statistically significant. For the rotarod performance, statistical analyses were undertaken by repeated measures two-way ANOVA.

RESULTS

The results of the stereological estimations are present as the dot plots (Fig. 3) which is a standard form of presentation of the data obtained using stereological methods. The total volume, number and length were shown in the rats of different groups. Each dot represents the parameter of an animal and the small horizontal bar indicates the average of the data. In this form of presentation the distribution of the data around the mean can also be observed.

Volumes of the cerebellum, cortex, and white mater

The study results revealed ~31% and 36% decrease in total volume of the cerebellum and cortex, respectively in the sodium metabisulfite-treated rats compared to the distilled water group (p<0.01). However, no significant change was observed in the white matter volume. The volume of the cerebellum and its cortex did not change in the sulfite+curcumin-treated animals in comparison to the distilled water-treated rats (Fig. 3A, B, C). This finding indicates the protective effects of curcumin.

Purkinje cell number

The findings of the study revealed ~19% decrease in the total number of the Purkinje cells in the sodium metabisulfite-treated rats compared to the distilled water group (p<0.01). The number of the Purkinje cells did not show any change in the sulfite+curcumin-treated animals in comparison to the distilled water-treated rats (Fig. 3D).

Mean total length of the nerve fibers

The mean total length of the nerve fibers per Purkinje cell was estimated in different groups (Fig. 3). The study findings revealed that the length of the nerve fibers in the cerebellar cortex was decreased by ~24% in the sulfite-treated animals in comparison to the distilled water group (p<0.01). However, no significant reduction was detected in their length in the sulfite+curcumin-treated rats in comparison to the sulfite-treated animals (Fig. 3E). The results also showed that curcumin or its vehicle (olive oil) did not affect the length of the fibers.

The fixed speed rotarod protocol

Fig. 4A summarizes the results of the protocol. The results of this experiment demonstrated that administration of sodium metabisulfite impaired (p<0.001) the rotarod performance that was significantly different from the other groups. In this experiment, all the animals gavaged with sodium metabisulfite showed a significant decrease in the latency to fall compared to the other groups.

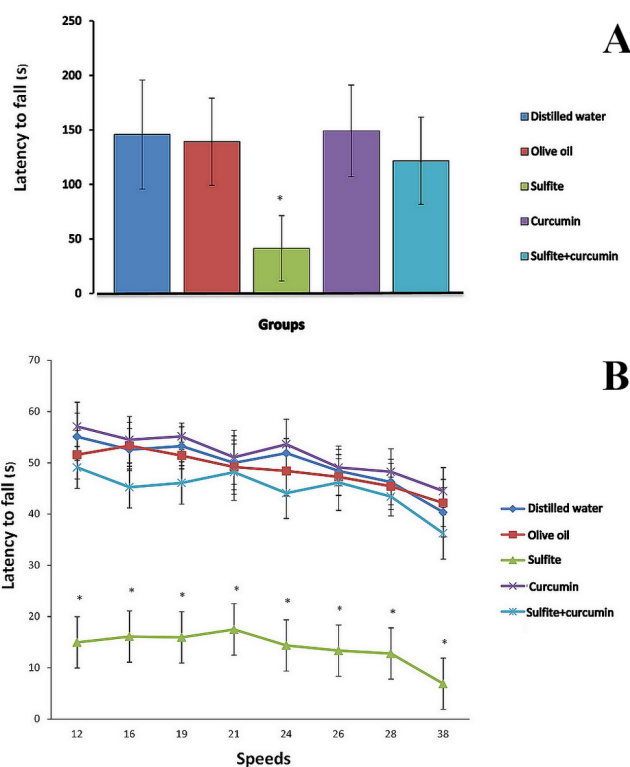In contrast, no significant difference was found between the

Fig. 4. Rotarod performance in the groups treated with distilled water, olive oil, sodium metabisulfite, curcumin and sodium metabisulfite+curcumin. (A) Fixed speed rotarod performance. Mean±SD of the latencies to fall in the different groups. It is shown for each of the eight fixed rotation speeds, tested at the end of experiments. (B) Accelerating rotarod performance. Mean ± SD of the latencies to fall in the different groups. *p<0.001, significant difference can be seen in sodium metabisulfite-treated with respect to distilled water and sodium metabisulfite+curcumin performance (n=10).

other groups. In fact, their performance was nearly stable at below 26 rpm speeds, leading to mean latencies to fall of about 45~60 s and the latency to fall was just slightly decreased at higher speeds; i.e., 26 to 38 rpm (Fig. 4A).

In addition, the present results showed that the sodium metabisulfite effect was significantly recovered in the rats treated with sulfite+ curcumin. This means that curcumin could protect the sodium metabisulfite-induced impairment of the rotarod performance.

The accelerating speed protocol

The results of accelerating protocol are presented in Figure 4B. A significant reduction in rotarod performance was observed in sodium metabisulfite group (p<0.001). In this protocol, the performance of the other groups did not significantly differ from the distilled water group and their mean latency to fall was approximately 120~150 s. Furthermore, the latency to fall was significantly increased in the rats treated with sulfite+curcumin compared to the sodium metabisulfite group.

DISCUSSION

Stereology is a popular technique to estimate the quantitative data of three-dimensional materials. It has helped to detect tissue structural changes in histo-pathological conditions. Considering the role of cerebellum in learning, cognition, and control of motor functions [20], evaluation of stereological parameters, including cerebellar tissues volume and Purkinje cell number, may be useful to uncover the histological changes in cerebellar disorders. The first step of the present work elucidated the effects of sulfite on the total volume of the cerebellum, cortex, and white matter, the total number of Purkinje cells and fiber length per purkinje in the sulfite-treated group. Our results indicated that all these parameters, except for white matter, were significantly decreased in the sulfite-induced group compared to the control group. The reduction in the total volume of the cerebellum and cortex can be attributed to the loss of Purkinje cells. These findings are consistent with those of a previous study showing the toxic effects of sulfite on the rats' mesencephalic cell line. These observations are in agreement with the previous studies showing the sulfite toxicity on neurons. For example, Akdogan et al. [4] showed loss of pyramidal neurons in the subdivisions of the rat hippocampus caused by sulfite treatment. The rotarod apparatus has been used to evaluate changes in motor function caused by some pharmacological agents, such as tranquilisers and antidepressants and antianxiety compounds [21, 22]. In the present study, the rotarod test was used to evaluate the effect of sodium metabisulfite on motor coordination. The results showed impairment of the performance

of the sulfite-treated rats in rotarod after 56 days. Furthermore, our previous work demonstrated that sulfite administration impaired learning and memory in rats [23].

It has been shown that neurotoxic effect of peroxynitrite radicals is potentiated by sulfite [6]. Although the mechanism of sulfite toxicity on the central nervous system is not completely understood, sulfur- and oxygen-centered free radicals may be responsible for this effect of sulfite [24]. Sulfite metabolism generates an intermediate product called sulfur trioxide radical that may act in some toxic effects of sulfite, including increment of lipid peroxidation, impairment of DNA synthesis, and destruction of amino acids [25]. It has also been indicated that the rats which possess normal sulfite oxidase enzyme activity can tolerate large amounts of sulfite and do not show toxicity [26]. It can be proposed that the harmful effects of sulfite can be attributed to cysteine-S-sulfate as a brain damaging metabolite which acts in sulfite oxidase enzyme deficiency. The human body does not have Cysteine-S-sulfate naturally [27]. It was found that rats have more ability in sulfite-oxidizing action compared to humans. It has also been reported that 750 mmol sulfite per kilogram body weight per day, equivalent to 48 g sulfite per kilogram body weight per day, is oxidized by sulfite oxidase enzyme in rats [28]. Moreover, some researchers have mentioned that various individuals have polymorphonuclear leukocytes with considerable variation in sulfite oxidase enzyme activity that is in agreement with the proposed polymorphic distribution of sulfite oxidase enzyme in humans [24-28]. Our results are confirmed by much evidence mentioned above about the detrimental effects of sulfite.

The doses of the sodium metabisulfite were prescribed according to the previous studies. Earlier studies have reported the acceptable daily intake of 163 mg/day by World Health Organization (WHO, 1994) from foods and drink in a single day or meal [2, 29, 30]. The acceptable daily intake of 0~0.7 mg/kg was assigned to sulfur dioxide and to sulfur dioxide equivalents arising from $Na_2S_2O_5$. Therefore, it should be noted that the mean per capita of sulfite intake limit from food and beverages is estimated as 19 mg sulfur dioxide equivalents per day. This level is reported to be 163 mg/kg sulfur dioxide equivalents in the 99th percentile of the population [2, 29, 30]. However no one knows correctly how much sulfite is ingested by the general population because it depends on dietary regime.

The dose of curcumin was selected according to our earlier results. Our previous study showed that the appropriate dose for the curcumin with no side effects on the liver, kidney and blood levels of aspartate aminotransferase, alanine aminotransferase, urea nitrogen and creatinine was 100 mg/kg/day [7, 31].

Another goal of the current study was to assess the possible

neuroprotective effect of curcumin. Our findings indicated that damaging sulfite effects were significantly reversed following curcumin administration in the fifth treatment group. In addition, curcumin treatment prevented the sulfite induced cerebellar damage as shown in Fig. 3 with no significant changes in the cerebellar volume and Purkinje cells number in the fifth group compared to the control group (distilled water). This observation shows the neuroprotective action of curcumin against sulfite. These findings are supported by our previous study showing the neuroprotective effect of curcumin on the dorsal root ganglion structure after sciatic nerve crush in rats. We also observed the functional and structural recovery by curcumin [8]. Another study has shown that curcumin has neuroprotective and anti-apoptotic effects on neurons following spinal cord injury [7]. Furthermore, other studies have reported that the regulation of the inflammatory cytokines, such as interleukin-6, tumor necrosis factor alpha, and cyclo-oxygenase-2, is modulated by curcumin. Therefore, curcumin shows anti-inflammatory effects [7]. Smith et al. [32] showed that the functional recovery after peripheral nervous system injury is prevented by early inflammatory events. Moreover, it was reported that neuroinflammation, excitotoxicity, cholinergic dysfunction, and oxidative stress in the cerebral cortex and hippocampus of okadaic acid treated mice were reduced as a result of curcumin administration. Also, memory function in both Morris water maze and passive avoidance tests was improved by curcumin administration in the mice with okadaic acid induced memory impairment [33]. In addition, studies have suggested that curcumin can improve the impaired memory of rats with chronic alcohol consumption [34]. It has been suggested that curcumin can modulate the release of serotonin and dopamine as an antidepressant. Moreover, the amount of neurotrophic factors, including brain derived neurotrophic factor, is elevated by curcumin [35]. Homocysteine neurotoxicity in the hippocampus of rats is inhibited by curcumin. The mechanism of protection appears to involve prevention of the formation of reactive oxygen species by curcumin in the rat's brain [36]. Curcumin is able to normalize the alterations caused by streptozotocin treatment in the cerebellum of rats [37]. Additionally, the findings of our previous study showed that curcumin had protective effects on the behavioral changes in chronic variable stress-induced rats [38]. Overall, the findings of the present study demonstrating the neuroprotective effect of curcumin are corroborated by the above-mentioned evidence.

Conclusion. Sulfite can induce changes in cerebellar cortex structure and functional changes in the rats' cerebellum and concomitant curcumin prescription plays a neuroprotective role.

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