Stereological studies of the effects of sodium benzoate or ascorbic acid on rats' cerebellum

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ABSTRACT

الأهداف: لتقييم هيكل المخيخ في بنزوات الصوديوم (NAB) أو حمض الاسكوربيك (AA) المعالج عند الفئران.

الطريقة: أجريت دراسة تجريبية خلال الفترة من مايو وسبتمبر 2013م في مركز أبحاث الحيوانات لجامعة شيراز للعلوم الطبية، شيراز، إيران. تلقت الفئران الماء المقطر، NAB (200mg/kg/day)، AA (100mg/kg/day)، و NAB + AA. أزيلت نصفي الكرة المخية بعد 28 يوما من حقنهم وخضعت للدراسة الكمية.

النتائج: حدث انخفاض فى حجم كل من نصف الكرة المخية، و قشرة المخيخ لها، وأنوية المخيخ الداخلية، والعدد الإجمالي من خلايا بيركينج وخلايا بيرغمان والخلايا الحبيبة والخلايا العصبية، والخلايا الدبقية في الطبقة الجزيئية ونوى الخلايا العصبية والخلايا الدبقية داخل المخيخ بنسبة 21-52% في الفئران المعالجة ببنزوات الصوديوم بالمقارنة مع مجموعة الماء المقطر (p=0.004) . وكان العدد الإجمالي للخلايا العصبية، وبيرغمان، وجولجي، والخلايا الحبيبية 29-45% أعلى في الفئران المعالجة بحامض الأسكوربيك بالمقارنة مع مجموعة الماء المقطر (p>0.05) . ومع ذلك فإن هذه القياسات تقل بنسبة 17-50% فى الفئران المعاملة ببنزوات الصوديوم مع حامض الأسكوربيك عند مقارنتها مع المجموعة الضابطة المعاملة بالماء المقطر (p=0.004) ولا تحدث أيضاً أي تغيرات شكلية محسوسة بالمقارنة مع المجموعة المعالجة ببنزوات الصوديوم .

الخاتمة: تركيب المخيخ يمكن أن يتغير بعد التعرض لبنزوات الصوديوم مع أو بدون العلاج لحامض الأسكوربيك. إلى جانب ذلك، يمكن لحامض الأسكوربيك منع فقدان بعض الخلايا في المخيخ.

Objectives: To evaluate the cerebellar structure in sodium benzoate (NaB) or ascorbic acid (AA) treated rats.

Methods: This experimental study was conducted between May and September 2013 in the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. The rats received distilled either water, NaB (200mg/kg/day), AA (100mg/kg/day), or NaB+AA. The hemispheres were removed after 28 days and underwent quantitative study.

Results: The total volume of the cerebellar hemisphere, its cortex, intracerebellar nuclei; the total number of the Purkinje, Bergman, granule, neurons, and glial cells of the molecular layer; and neurons and glial cells of the intracerebellar nuclei reduced by 21-52% in the NaB-treated rats compared with the distilled water group (p=0.004). The total number of the Purkinje, Bergman, Golgi, and granule cells was 29-45% higher in the AA-treated rats compared with the distilled water group (p=0.05). However, these measures reduced by 17-50% in the NaB+AA-treated rats compared with the distilled water group (p=0.004). The NaB+AA group did not induce any significant structural changes in comparison with the NaB group (p>0.05).

Conclusions: The NaB exposure with or without AA treatment could alter the cerebellum. Yet, AA could prevent the loss of some cells in the cerebellum.

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Sodium benzoate (NaB) is the sodium salt of benzoic acid and is widely used as a food preservative and antimicrobial substance in a variety of products, such as salads, pickles, vinegar, carbonated drinks, jams, fruit juices, and sauces. It is also used as a preservative agent in medications, cosmetic compounds, and shampoos.[1] The Food and Drug Administration (FDA) has limited addition of NaB as a preservative to 0.1% by weight.[2,3] Sodium benzoate is also allowed as an animal food additive up to 0.1% according to the World Health Organization (WHO) official publication.[3] It has been shown that NaB can be found as a metabolite of cinnamon.[2,3] Moreover, NaB is used for urea cycle disorders, suppresses inflammation, and switches the differentiation of T cells.[2,3] Several reports have also emphasized the neuro-pharmacological properties of NaB, including relief of panic symptoms, and protection of astrocytes and neurons protein in Parkinson disease, as well as the anti-inflammatory effect of NaB on microglia and astrocytes in multiple sclerosis.[4,5] A previous study showed alterations in rats' behavioral activities, such as motor impairment and anxiety after NaB treatment.[6] Some studies have also suggested that a high intake of NaB might be associated with attention deficit-hyperactivity disorder (ADHD).[6]

Ascorbic acid (AA) is one form of vitamin C. It is a water-soluble vitamin and can be found as a white or slightly yellow crystal or powder with a minor acidic taste. It has been shown that AA plays an essential role in maintaining motor abilities in mice.[7] It was also reported that plasma AA levels were lower in the subjects with dementia compared with the controls, and their data supported the free radical theory of oxidative neuronal damage.[8] Furthermore, it has been explained that benzene might be produced in soft and fruit drinks, which might be associated with the combined presence of AA and NaB ingredients in the products. However, benzene production may need a transition-metal catalyst.[9] Bonaccorsi et al[9] determined the amount of benzene in the soft drinks in Italy.

The cerebellum is important for making postural adjustments to maintain balance. It is also involved in coordination of voluntary movements, motor learning, and fine-tuning of motor programs. It may also play a role in ADHD.[10] The present study was designed to find quantitative responses to the following questions using stereological methods: How much does the rats' volume of the cerebellum, cortex, and intracerebellar nuclei change after treatment with NaB with or without AA? Does the number of Purkinje, Bergman, Golgi, granules, neurons, and glial cells of the molecular layer and the nuclei change after treatment by NaB with or without AA?

Methods. In this study, 24 Sprague-Dawely adult male rats weighing 170-230 g were sampled from the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. Ethical approval for the experiment and animal use was obtained from the Ethics Committee of the University. All efforts were made to diminish animal suffering. The animals were housed under standard conditions in plastic cages under a 12 hours/12 hours light/dark cycle at 22±2ºC.

The rats were randomly divided into 4 groups (n=6). The sample size was selected according to the standard stereological article.[11] Group I served as the control group receiving distilled water, while the other 3 groups were given NaB (200 mg/kg/day) (NegarAzar, Qom, Iran) dissolved in distilled water, AA (100 mg/kg/day) and NAB+AA by gavages.[12,13] The dose of NaB used in the current study was selected according to the study by Oyewole et al.[12] According to their outcomes, this dose induced neurological damage in the rats.[12] The treatments were continued for 28 days. At the end of the treatments, the right cerebellar hemispheres were removed.

Exhaustive coronal sections with 26 µm thickness from the right hemispheres were stained with cresyl violet. A total of 10-12 sections were sampled in a systematic uniform random sampling manner to estimate the total volume of the cerebellar hemisphere and cortex. Another set including 10-12 sections was also sampled containing intracerebellar nuclei. The lateral, interposed, and medial nuclei were considered collectively. Using a projecting microscope, the volume was estimated at the final magnification of 25× by Cavalieri's principle.[14] The distances between the sampled sections (d) were also calculated. In addition, the area was evaluated using a point-counting method. The area per point (a/p) was 0.83 mm^2 and 0.15 mm^2 for estimation of the volumes of the hemispheres and intracerebellar nuclei. On average, 110-220 points ($\sum P$) were counted per animal. Finally, the volume was computed using the following formula:

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

A computer attached to a light microscope (Nikon E200, Tokyo, Japan) and immersion lens (60×, numerical aperture: 1.4) was used to estimate the total number of the cells using the optical disector method.[14] The microscopic fields were sampled by moving the

microscope stage in an equivalent interval using a stage micrometer.[14] Using a microcator (MT12, Heidenhain, Traunreut, Germany) connected on the stage, the Z-axis movement of the microscope stage was measured.[14] The area (*a/f*) of unbiased counting frame was measured as 846 µm^2 for Purkinje, Bergman, Golgi, molecular layer neurons, and glial cells, 360 µm^2 for granule cells, and 1300 µm^2 for neurons and glial cells of the nuclei. A frame with accepted (right and upper) and forbidden (left and lower) borders was super-imposed on the images of the tissue sections viewed on the monitor. To analyze the appropriate guard zone and the height of the disector (h), Z-axis distribution of the nuclei was plotted.[15] The counted cells were scored and grouped in 10 bins from the percentiles 0-100 through the histological tissue section from the upper (0%) to the lower surface (100%). **Figure 1** shows the Z-axis dispersion of the cell nuclei. The upper and lower 30% of the histogram were considered as the guard zones and counting was carried out at the remaining 40% (h). According to the histogram, under-sampling was balanced out and corrected.[15] Any cell nucleus coming into the focus within the sampling box (h multiplying *a/f*) was selected if it was located totally or to a degree inside the counting frame and did not touch the forbidden line (**Figure 1**). The total number of the cells was estimated by multiplying the numerical density (Nv) by the total volume of the cortex or intracerebellar nuclei.

$$Nv(cells\ /\ unit\ volume) = \frac{\sum Q^-}{\sum P \times \left(\frac{a}{f}\right) \times h} \times \frac{t}{BA}$$

Where "ΣQ" was the number of the nuclei coming into focus during scanning the "h" (the height of the disector) (**Figures 2 & 3**)(On average, 350 cells were counted per animal), "ΣP" was the total number of neurons in the unbiased counting frame in all fields, "h" was the height of the disector, "*a/f*" was the frame area, "*t*" was the factual 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.[14] Using point counting, the coefficient of error (CE) for the estimate of the volume 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:

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

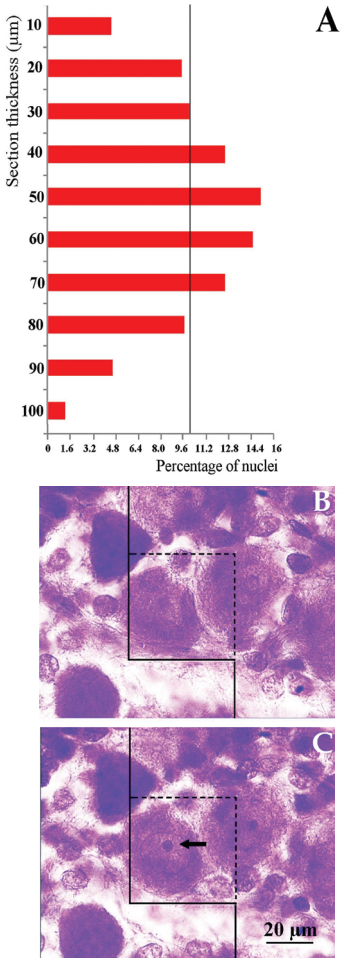

Figure 1 - Optical disector showing **A)** Z-axis distribution of the cerebellar cells. The upper and lower 30% of the histogram were the guard zones and the counting was carried out at the remaining 40% (height of the disector). **B & C)** The cell is considered for counting if nucleolus dose not appear in the look up section, can be seen in the reference section, and does not hit the left and lower borders of the frame (arrow).

Where "b" represented the mean section boundary length and "a" the mean sectional area.[16,17] The data were entered into the Statistical Package for Social Sciences software (SPSS Inc., Chicago, IL, USA) version 16.0 and analyzed using Kruskall-Wallis and Mann-Whitney u-test. A value of $p<0.05$ was considered statistically significant.

Results. *Sodium benzoate-treatment.* The total volume of the cerebellar hemisphere and its cortex reduced by 26% in the NaB-treated rats in comparison

with the distilled water group (p=0.004). In addition, the total number of the Purkinje reduced by 21%, Bergman by 49%, granule by 49%, neurons by 49%, and glial cells of the molecular layer by 42% in the NaB-treated rats in comparison with the distilled water group (p=0.004) (**Figures 2 & 4**). The total volume of the intracebellar nuclei of the hemisphere was reduced by 22% in the rats treated with NaB in comparison with the distilled water-treated group (p<0.05). The total number of the neurons of the intracebellar nuclei decreased by 43% and glial cells by 52% in the rats treated with NaB in comparison with the distilled water-treated group (p=0.004) (**Figure 3**).

Ascorbic acid treatment. The results of the present study indicated no significant difference between the AA-treated rats and the distilled water group regarding the total volume of the cerebellar hemisphere and its cortex. Additionally, the total number of the Purkinje (29%), Bergman (45%), Golgi (30%), and granule cells (23%) was higher in the AA-treated rats compared with the distilled water group (p=0.05) (**Figures 2 & 4**).

The total volume of the intracebellar nuclei of the hemisphere and the total number of the neurons and glial cells in the nuclei did not show any significant

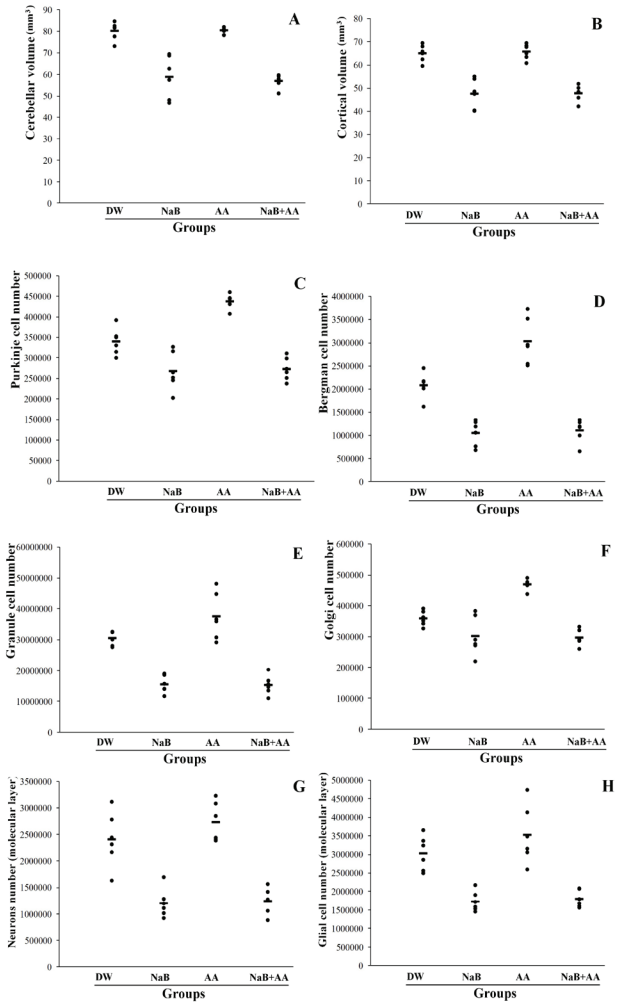

Figure 2 - The scatter plots of the **A**) total volume and **B**) cortex of the cerebellar hemisphere, and the total number of the **C**) Purkinje, **d**) Bergman, **e**) granule, **F**) Golgi, **G**) neurons, and **H**) glial cells of the molecular layer of the animals treated with distilled water (DW) and sodium benzoate (NaB) with or without ascorbic acid (AA). Each dot represents an animal and the horizontal bar is the average of values of animals in each group.

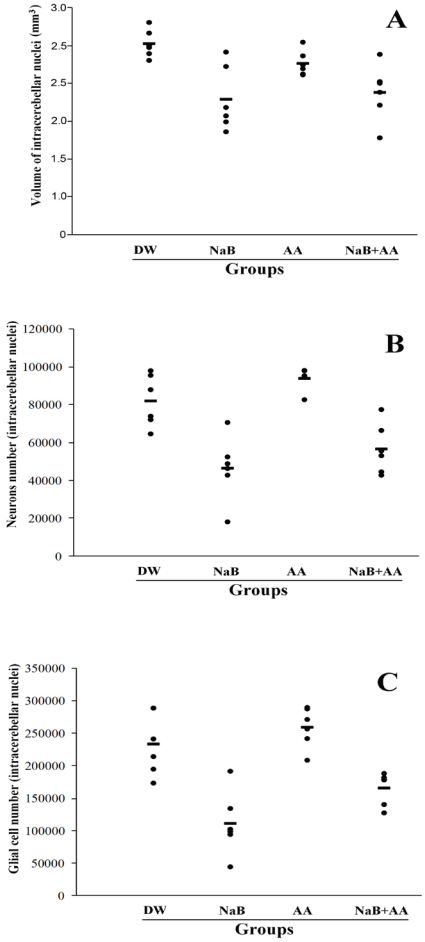

Figure 3 - The scatter plots of the **A**) total volume and **B**) the total number of the neurons, and **C**) glial cells of the intracerebellar nuclei of the animals treated with distilled water (DW) and sodium benzoate (NaB) with or without ascorbic acid (AA). Each dot represents an animal and the horizontal bar is the average of values of animals in each group

differences in the AA-treated rats in comparison with the distilled water group (p>0.05) (**Figure 3**).

Sodium benzoate + ascorbic acid treatment. The total volume of the cerebellar hemisphere reduced by 29% and its cortex by 26% in the NaB+AA-treated rats in comparison with the distilled water group (p<0.004). Moreover, the total number of the Purkinje of the molecular layer reduced by 20%, Bergman by 50%, Golgi by 17%, granule by 50%, neurons by 48%, and glial cells by 40% in the NaB+AA-treated rats compared with the distilled water group (p=0.004) (**Figures 2 & 4**).

The total volume of the intracebellar nuclei of the hemisphere was reduced by 25% in the rats treated with NaB+AA in comparison with the distilled water-treated group (p=0.05). The total number of the neurons decreased by 31% and glial cells of the intracebellar nuclei by 29% in the rats treated with NaB+AA compared with the distilled water-treated group (p=0.05) (**Figure 3**). Treatment of the animals with NaB+AA did not induce any significant structural changes in comparison with the NaB group (p>0.05).

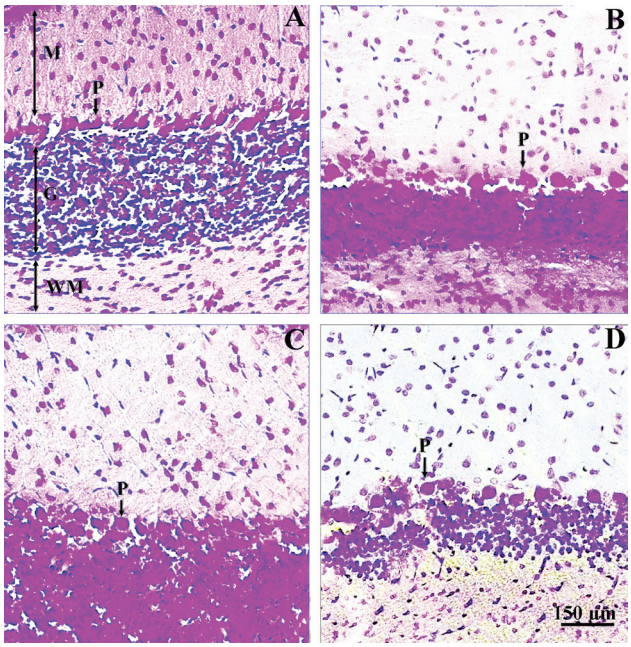

Figure 4 - The effects of treatment of the rats with **A**) distilled water, **B**) sodium benzoate (NaB), **C**) ascorbic acid (AA), and **D**) NaB+AA on the cerebellar histology. M - molecular layer, P - Purkinje cells, G - granular layer, WM - white matter. Loss of the Purkinje cells can be seen in the NaB-treated animals. More population of the Purkinje cells can be observed in the AA-treated rats. No remarkable differences can be detected between NaB- and NaB+AA treated rats regarding the population of the Purkinje cells.

Discussion. The present study evaluated the quantitative histological changes of the rats' cerebellum after treatment with NaB and AA using stereological methods. According to the Joint FAO/WHO Expert Committee on Food Additives, the acceptable daily intake level for NaB and potassium benzoate is 0-5 mg/kg body weight.[3] However, the exact amount of NaB taken by individuals cannot be determined exactly and depends on the daily received fast foods, salads, jams, and other foods containing the preservative. The current study tried to evaluate the effects of NaB on the cerebellum. The results revealed an extensive reductive change in the structure of the cerebellum after NaB-treatment. The cerebellum plays a role in motor and cognition functions.[10] Previous research showed a relationship between NaB intake by children and onset of hyperactive behavior.[1] In addition, our previous report confirmed the impairment in the motor function of the rats treated with NaB.[18] Furthermore, exposure of zebrafish embryos to NaB led to phenotypic malformation.[1] Thus, reports of motor impairment, ADHD, and cognitive function impairment might be explained using the findings of the present study.

A possible mechanism of the effect of NaB might be changes in the neurotransmitter levels. It has also been shown that NaB can increase the gamma-amino butyric acid levels and reduce the level of zinc in the brain.[19] Therefore, neurotransmitter alteration might be one of the possible mechanisms of behavioral or phenotypic changes. The other mechanism of action of NaB can be explained by the oxidative stress process. It has been reported that malondialdehyde increased in human erythrocytes after NaB exposure in vitro.[20] This reactive species naturally exists in cells and tissues and is an indicator of oxidative stress. Glutathione peroxidase is also decreased in the erythrocytes after NaB exposure in vitro.[20] Glutathione peroxidase is an enzyme with peroxidase activity whose main biological task is to protect the organism from oxidative injury. The findings of the study by Yetuk et al[20] suggested that NaB might induce its side effects on the cerebellum by increasing oxidative stress in the nervous tissue.

The effects of AA alone or combined with NaB on the cerebellum were also evaluated. The NaB might be used in the dietary products including AA. The AA is an essential vitamin in the nervous system and has anti-oxidative properties.[21] The findings of this study showed that the number of some cerebellar cells was higher in the AA-treated rats compared with the distilled water group. There are many studies that support the protective action of AA. It has been reported that the plasma level of AA was lower in the individuals with

dementia compared with the controls.[8,22] Further nourishing with this vitamin may stop or impede the progression of cognitive impairment in patients with Alzheimer's disease.[8,22] It has been reported that vitamin C supplementation could protect neurons and astrocytes in the cerebellar cortex of rats during neurotoxic and degenerative conditions.[22] It has also been shown that AA was able to block the apoptotic process due to the formation of reactive oxidative species during aging.[23] Moreover, a study indicated that postnatal AA insufficiency in guinea pigs resulted in impaired spatial memory task, significant decrease in the number of neurons, and reduction of the volume of the hippocampus and the dentate gyrus.[23] A possible protective mechanism of AA can be explained by donating electrons to deactivate many free radicals and playing a role in regeneration of other antioxidants in biological tissues.[21-24] The AA also helped maintain the activity of tyrosine hydroxylase by recycling its essential co-factor and also stimulated catecholamine synthesis.[21-24]

It should be noted that neurons cannot be regenerated in most parts of the rats' CNS. Therefore, the higher cell number in the AA-treated rats of the current study can be related to the protective effects of AA on the cells that might be lost during aging. It was found that the number of Purkinje cells in the sedentary rats aging more than 12 weeks was lower than that in the adult ones.[25,26] The cerebellum produces the highest levels of nitric oxide within the CNS.[25,26] High levels of nitric oxide may make the cerebellum more vulnerable to oxidative stress.[25,26] Aging is the outcome of the damaging effects of free radicals produced during cell metabolism and reactive oxygen species, such as nitric oxide. Therefore, AA might have exerted a protective agent in age-related cell loss in the cerebellum in the present study. This finding can be followed in future research on aging.

When AA was prescribed with NaB, no protective change was detected. This might be due to the high levels of reactive oxygen species that could not be controlled by AA. In addition, it has been mentioned that a concentration of benzene can be found in the beverages containing both AA and NaB.[9] Therefore, if benzene was produced after NaB+AA-treatment, AA could not play its protective role.

One of the limitations of the study was lack of immunohistochemical detection and quantification of different types of cell death including apoptosis, necrosis, and autophagy. The findings of the present study can be followed by future research detecting the type of cell death and the related mechanism after NaB consumption. The mechanism of action of AA should also be assessed.

In conclusion, the structure of the cerebellum can be altered after NaB with or without AA-treatment in rats. The volume of the hemisphere, cortex, and intracerebellar nuclei and number of the cells in the cortex could be reduced in the NaB-treated rats. Ascorbic acid could prevent the loss of Purkinje, Bergman, Golgi, and granule cells in the cerebellum. Nonetheless, co-treatment with NaB and AA could not prevent the cell loss.

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