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CHANGES IN S100 PROTEIN EXPRESSION IN THE CEREBELLUM OF RATS ORALLY EXPOSED TO ENERGY DRINKS

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Abstract

The aim of our study was to assess the rate of S100 protein expression in the cerebellum of rats orally exposed to caffeinated energy drinks (CED) during 2 and 4 weeks. Twenty WAG rats were enrolled in the experiment. They were subdivided into three groups. The animals from group 1 and group 2 (n=7 in each) were orally exposed to a caffeinated energy beverage "Black" at a dose of 12 ml per kg of body weight during two and four weeks, respectively. The control group consisted of 6 intact rats obtained drinking water instead of a caffeine-containing energy drink. Cerebellar S100 expression was evaluated immunohistochemically. Against the background of CED oral administration during two and four weeks, the density of granule cell layer becomes lower indicating neuronal loss. S100 protein was upregulated in group 1 in the entire cerebellar cortex. Thus, CED oral exposure resulted in the development of cerebellar astrogliosis. Four-week-long CED consumption resulted in S100 downregulation in the molecular and granular layers. Our findings demonstrate the damage to the cerebellum in rats exposed to CEDs with overexpression of S100, followed by a decrease in its expression.

INTRODUCTION

The consumption of caffeinated energy drinks (CEDs) has been significantly increasing over decades worldwide [1]. The market offers a vast diversity of various both alcoholic and non-alcoholic energy beverages manufactured by dozens of brands. Non-alcoholic CEDs may contain over a dozen of ingredients, including caffeine, whose content can reach 505 mg per a small bottle (250 ml), taurine, L-carnitine, vitamins B, ginseng, guarana, sugar, glucuronolactone, etc [2].

Converging lines of evidence indicate that the oral exposure to non-alcoholic CEDs has been associated with numerous adverse effects [3-5]. In particular, Hammond et al (2018) stated that the side effects of energy drinks were consistent with those caused by the high caffeine intake. However, they were more pronounced compared with those developed as a result of caffeine consumption from coffee [6]. Given that caffeine affects the central nervous system (CNS) and caffeinated energy beverages are used to improve the mental productivity, it is of huge importance to assess their impact on the CNS.

There is some evidence that CEDs influence morphological and metabolic parameters in the CNS. In particular, Salih et al (2018) reported that the oral consumption of CEDs by rabbits led to the following morphological changes in the brain in a dose-dependent manner: reduction of nerve cells, focal gliosis; some neurons were shrunken and contained pyknotic nuclei [7]. It has been also demonstrated that long-term oral consumption of CEDs results in the activation of regenerative processes in the brain, evidenced by Ki-67 overexpression [8]. In addition, the reduced content of brain-derived neurotrophic factor (BDNF) was found in the brain against the background of CED oral exposure [8].

It has also been reported that consumption of caffeine-containing beverages may affect the cerebellum [9]. However, the number of papers that focus on the impact of commercially available CEDs on the cerebellum is limited. Thus, little is known about changes in the cerebellar tissue observed under the influence of CED oral intake. Therefore, our research was aimed at evaluating the rate of S100 protein expression in the cerebellum of rats orally exposed to caffeinated energy drinks during 2 and 4 weeks, respectively.

MATERIALS AND METHODS

Experiment design and groups of animals

Twenty adult female WAG rats whose weight ranged from 180 to 200 g were enrolled in the experiment. They were provided by the vivarium of Kharkiv National Medical University. All animals were randomly subdivided into three groups. The rats from group 1 (n=7) and group 2 (n=7) were daily orally exposed to a caffeinated energy drink "Black" (12 ml/kg of body weight) during two and four weeks, respectively. The drink contained 320 mg/l of caffeine, guarana extract, taurine, and vitamins B. On Sundays the beverage was not given. The control group (n=6) consisted of intact animals obtained drinking water instead.

Bioethics

The design of our research and all manipulations with the animals enrolled were approved by the Committee of Ethics and Bioethics (Kharkiv National Medical University). The study was performed in strict accordance with the EU Directive 2010/63/EU (September 22, 2010), which was based on the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123, 1986).

Routine staining techniques

The animals were anesthetized and sacrificed. Then the cerebellum was collected. Fragments of cerebellar tissue were fixed in formalin solution. After fixation, samples were dehydrated via the use of ascending grades of alcohol. The tissues were used to prepare paraffin-embedded tissue blocks. These blocks were used to obtain 4-μm-thick sections with the help of a microtome. Then the sections were stained by hematoxylin & eosin and Einarson's gallocyanin-chrome alum.

Immunohistochemical study of S100 expression

Immunohistochemical staining was performed with primary mouse monoclonal antibodies to S100 protein purchased from *Thermo Fischer Scientific* (UK). Then according to the staining protocol, the samples were incubated with the secondary horseradish peroxidase-conjugated secondary antibodies. As a chromogenic substance for visualization, 3,3′-Diaminobenzidine (DAB) was used. The positive DAB staining was brown.

RESULTS

The cerebellar cortex of the animals from the control group stained with hematoxylin & eosin and Einarson's gallocyanin-chrome alum consisted of three layers: molecular, Purkinje cells, and granular (**Figures 1a, 2a**). The Purkinje layer located between the molecular and granular ones is composed of a monolayer of large pear-shaped neurons called Purkinje cells. The outermost molecular layer is cell-poor, whereas the cell-rich innermost granular layer is comprised of small-sized interneurons referred to as granule cells (**Figures 1a, 2a**). As a result of CED consumption in rats from group 1, the density of granule neurons in the cerebellum decreased (Figures 1b, 2b). Changes were also found in the Purkinje layer. The Purkinje neurons were surrounded by glial cells whose number was visually higher than in the control group.

The intake of CEDs during 4 weeks (group 2) resulted in some morphological changes in the cerebellum. In particular, hollow spaces were found in the Purkinje and granule cell layer (Figures 1c, 1d). Such lower density indicates the neuronal loss. Furthermore, the Purkinje neurons in many regions of the cerebellum looked smaller. Many of them became oval, while in the control group they were pear-shaped (Figure 1a). Moreover, the Purkinje neurons seemed to be less visible than in controls. In this study, we found that the Purkinje cells in rats from group 2 were surrounded by numerous glial cells that could be observed on the body of neurons. Figures 1c, 1d, 2c and 2d show images with the Purkinje cells being "eaten" by microglial cells. This was not observed in the control group.

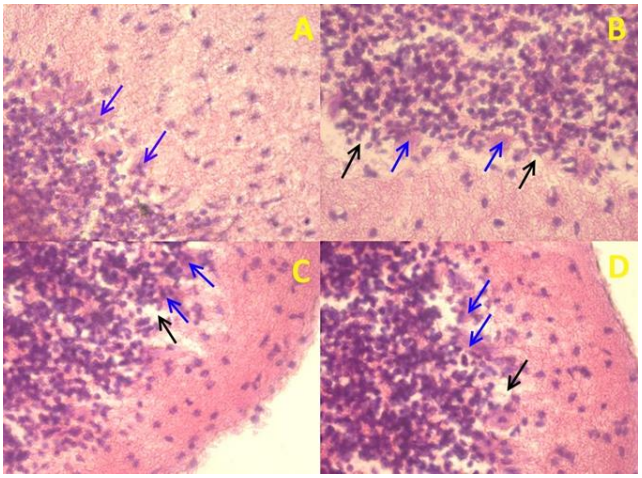

Figure 1. Sections of cerebellum stained with hematoxylin & eosin. A). Section of cerebellar tissue of a rat from the control group. Intact granular and molecular layers with Purkinje neurons (blue arrows) between them can be seen. 400x. B) Representative image of cerebellum of a rat from group 1. Purkinje neurons (blue arrows) are surrounded by gliocytes. The number of glial cells is higher than in the control group. Hollow spaces are found (black arrows). 400x. C and D) Representative images of cerebellar cortex from the rats of group 2. Hollow spaces can be seen in the cerebellum (black arrows). Purkinje cells (blue arrows) seem to be smaller than in controls. Glial cells are localized on the body of the Purkinje cells. 400x

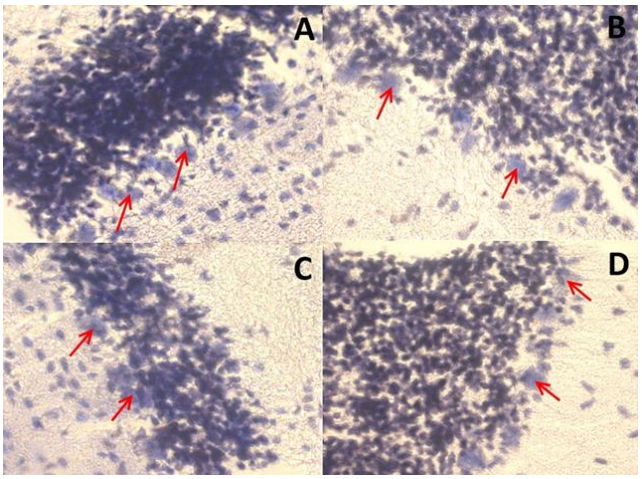

Figure 2. Sections of cerebellum stained with Einarson's gallocyanin-chrome alum. A). Control group cerebellar cortex with the distinctly visible layers: molecular, Purkinje, and granular. Purkinje neurons are marked with blue arrows. The granule cell layer is dense. 400x. B). Cerebellar tissue of an animal from group 1. Purkinje neurons are surrounded by numerous glial cells (red arrows). The granular layer is less

dense, indicating granule neuronal loss. 400x. C and D). Cerebellum of the rats from group 2. Pear-shaped Purkinje cells became oval-shaped (red arrows). They are poorly visible and surrounded by glial cells. The granule cell layer is sparser than in the control group. 400x.

Analysis of immunostaining in the control group showed that the weak S100 expression was observed in many cells in the molecular layer of cerebellum. It is worth noting that the expression of S100 is distributed diffusely. S100-positive cells with mild and moderate immunostaining were found in the granular layer. We also revealed S100-labeled cells in the Purkinje layer, located between the molecular and granular layers (**Figure 3**). They are presumably Bergmann glial cells.

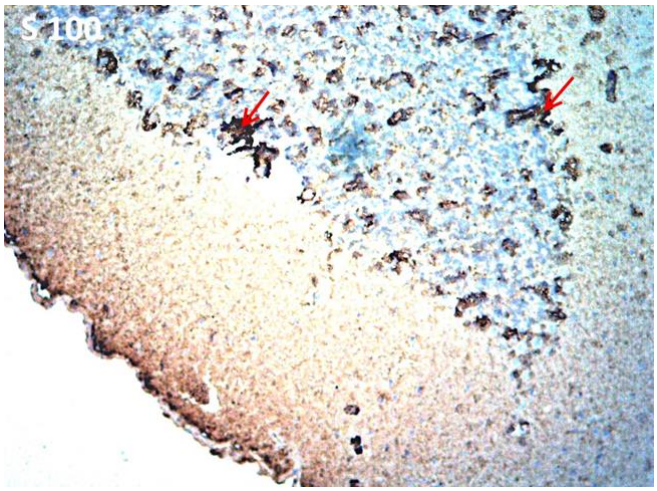

Figure 3. Cerebellum of a rat from the control group. S100 immunostaining is observed in all cerebella layers. S100-positive glial cells are marked with red arrows. Immunohistochemical reaction with antibodies to S100 protein. 100x.

Analysis of cerebellar S100 expression in rats from group 1 (orally exposed to CEDs during two weeks) demonstrated that the molecular layer was characterized by a more pronounced diffuse S100 immunostaining with a higher number of S100-positive cells compared with the control group. Furthermore, the intensity of expression was found to be higher. The same situation was observed in the granular layer. It contained more S100-labelled glial cells in animals from group 1 than in controls (**Figure 4 a-d**). We observed some foci of various sizes and shapes in the granular layer with the strong S100 expression. Such formations in the Purkinje cell have immunostained cell processes. It is worth mentioning that S100-positive foci contained several immunostained cells. In regions of the cerebellar granular layer located between the S100-positive foci, there were spaces with glial cells with low S100 expression (**Figure 4 a-d**). In general, the glial cells with diverse expression intensity dominated.

S100 expression was also evaluated in the cerebellum of rats from group 2 (orally consumed CEDs during four weeks). The molecular layer was characterized by the diffuse S100 expression. However, S100 was found to be downregulated compared with controls (**Figure 5 a-d**). Foci with S100-positive cells were not observed in the molecular layer in group 2. It is important to note that solitary groups of S100-labeled glial cells can be seen in the granular layer. S-100 positive Bergmann cells were found in the Purkinje layer. However, the expression of astrocyte-derived S100 protein in them was lower than in the control group. It is worth mentioning that hollow spaces were revealed in the Purkinje layer. S100 expression in the granular layer was less pronounced compared with controls.

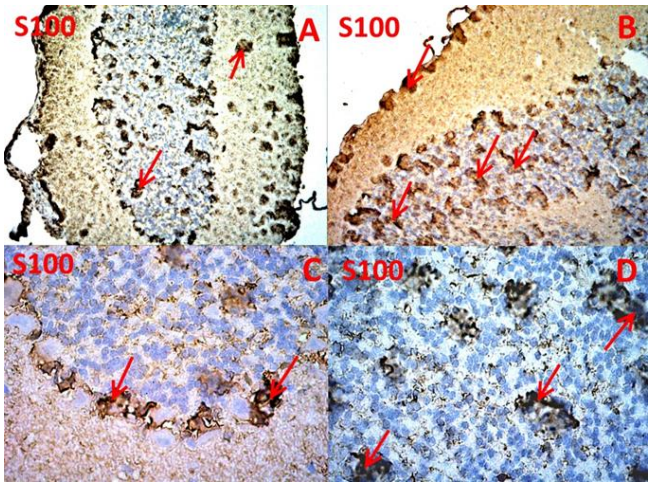

Figure 4. Cerebellar immunostaining. Microslides of animals from group 1 exposed to caffeinated energy drinks during two weeks. Immunohistochemical reaction with antibodies to S100 protein. A) S100 immunostaining is stronger in all layers of the cerebellum than in controls. Strong immunolabelling is marked with red arrows. 100x. B) S100 overexpression is revealed in all layers of the cerebellum compared with controls. S100-positive glial cells are marked with red arrows. 100x. C). S100-positive cells are found in the Purkinje layer (marked with red arrows). 400x. D) Strong S100 expression is observed in the cerebellum (marked with red arrows). The amount of S100-positive gliocytes in the granular layer (marked with red arrows) is higher compared with the control groups. 400x.

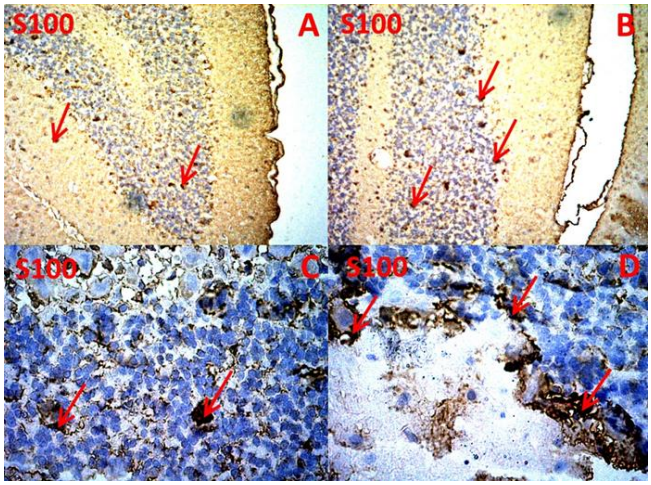

Figure 5. Cerebellar immunostaining. Cerebellar immunostaining. Microslides of animals from group 2 orally exposed to caffeinated energy drinks during one month. Immunohistochemical reaction with antibodies to S100 protein. A) S100 is downregulated in the cerebellum. The number of S100-labelled cells is lower in the granular layer than in the control group (marked with red arrows). 100x. B) Low amount of S100-positive glial cells can be seen in the granular layer (marked with red arrows).

100x. C). Solitary S100-positive cells are revealed in the granular layer (marked with red arrows). 400x. D) In the Purkinje layer, we can notice several S100-positive cells, presumably they are Bergmann glial cells (marked with red arrows). They are characterized by strong S100 immunostaining. 400x.

DISCUSSION

S100 proteins are a family of small, acidic, calcium-binding, multifunctional proteins that includes 25 representatives and are responsible for the regulation of cell differentiation, proliferation, inflammation, apoptosis, migration, energy metabolism, etc [10, 11]. It has been reported that S100 proteins are expressed under normal circumstances in the cerebellum. In particular, S100 is expressed in Bergmann glial cells, which are unipolar astrocytes that surround Purkinje cells, and astrocytes [12, 13]. However, S100 expression is not observed in cerebellar neurons, such as granule cells and Purkinje cells. Furthermore, S100 is recognized as a biomarker of astrocytic activation and its upregulation is observed in response to injury and brain damage associated with the loss of neurons [14]. Thus, S100 overexpression found in this study in the rats from group 1 indicates the activation of astrocytes (astrogliosis), including Bergmann glial cells (Figure 4c), in response to cerebellum damage caused by CEDs, evidenced by neuronal loss observed when analyzing the routinely stained microslides of cerebellum. Astroglyosis development has a protective purpose, since reactive astrocytes are involved in repair of the nervous tissue [15]. However, it may also have detrimental effects. There is strong evidence that reactive astrocytes may promote inflammatory responses [16, 17]. When S100 protein is released from destroyed astrocytes, it can bind to receptors for advanced glycation end products (RAGEs), activating the NF-κB pathway, which is associated with the synthesis of pro-inflammatory cytokines TNFα and IL-1β, as well as iNOS [18, 19]. Thus, it is hard to say whether the neuroprotective effects of astrogliosis observed as a response to CED consumption outweigh its negative effects.

The consumption of CEDs resulted in S100 downregulation, which might be indicative of the reduced stress resistance. Such changes in S100 expression can be considered to be a sign of the reduced number of reactive astrocytes. As a result, neurons can become more vulnerable due to the long-term oral exposure to CEDs. It is worth mentioning that S100 prevents apoptosis of neurons [11]. Therefore, its downregulation in the cerebellum of rats from group 2 may contribute to the neuronal cell death. This hypothesis is confirmed by less dense regions in the granule cell and Purkinje layers and the presence of more hollow spaces in them in group 2 compared with group 1, indicating a more severe neuronal loss in rats exposed to CEDs longer. We believe that continuous astrocyte astrocyte activation in response to ingredients of CEDs exhausted glial cells, which negatively affected neuronal survival and promoted cell death. We speculate that such dead Purkinje neurons probably get phagocyted by microglia like it can be noticed in Figures 1c, 1d, 2c, 2d.

Our research had some limitations. First, we did not assess S100 mRNA expression in astrocytes. However, immunostaining clearly indicates upregulation of S100 against the background of CED consumption. Second, we did not have the opportunity to microglial-specific markers like Iba-1 to confirm our hypothesis on phagocytosis of Purkinje neurons by microglia.

Our study raises concerns over the safety of CEDs and substantiates the relevance of novel researches that aim at elucidating the toxicity profile of CEDs.

CONCLUSIONS

Our findings suggest that the prolonged oral intake of CEDs by rats causes damage to the cerebellum with a decrease in the amount of granule cells indicating loss of granular neurons. Two-week-long exposure to CEDs is associated with overexpression of astrocyte-derived S100 protein in all three layers of the cerebellar cortex, which is indicative of astrogliosis. Four-week-long CED consumption resulted in S100 downregulation in the molecular and granular layers. The Purkinje cells are damaged as a result of CED consumption, evidenced by the change in the shape of cell body and accumulation of glia around them.

DISCLOSURE

All authors have contributed to the manuscript equally. None of the authors have direct or financial conflicts of interest with this paper and material contained herein.

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