ORAL CONSUMPTION OF CAFFEINATED ENERGY DRINKS INCREASES EXPRESSION OF KI-67 BUT DECREASES BRAIN-DERIVED NEUROTROPHIC FACTOR IN THE BRAIN OF RATS.

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ABSTRACT

Cytotoxic effects of caffeinated energy drinks and their components have previously been reported. **The aim** of this study was to investigate the effect of regular oral consumption of caffeinated energy drinks on the brain, especially on cerebral Ki-67 expression, levels of brain-derived neurotrophic factors and β-nerve growth factor in a rat model. **Materials and methods.** The brain homogenate levels of BDNF and β-NGF were measured in rats fed on a regular diet of caffeinated energy drinks for two months, using commercially available ELISA kits and compared with levels measured in control rats. Ki-67 expression was assessed immunohistochemically to evaluate the proliferation rate of different types of cells in the brain tissue. **Results.** Caffeinated energy drink consumption for two months was associated with the development of histological features of brain edema, significantly increased expression of Ki-67 with BDNF levels significantly reduced by half (p=0.02), in brain homogenates compared to control rats. **Conclusions.** Chronic oral consumption of caffeinated energy drinks was associated with significantly reduced BDNF levels and an overexpression of Ki-67 in macroglial cells, endotheliocytes, and ventricular ependymocytes suggesting a proliferative response to neural injury.

Key words: Brain-Derived Neurotrophic Factor, β-Nerve Growth Factor, Caffeine, Energy Drinks, Ki-67 Antigen.

INTRODUCTION

Caffeine is considered to be the most widespread stimulant consumed worldwide. [1] It is known that caffeine improves mood, increases physical and mental performance and endurance. [2] In addition to its traditional sources such as tea and coffee, nowadays caffeine is widely added to various kinds of beverages, including caffeine-containing energy drinks. [1, 3, 4] Energy beverages contain much higher concentrations of caffeine in comparison with natural stimulating drinks. It has been reported that some energy drinks may have up to 500 mg of caffeine per serving, whereas the Food and Drug Administration recommends its daily consumption should be limited to 300 mg. [3, 5] Due to the diversity of caffeine dietary sources, its consumption has recently skyrocketed. Caffeinated energy

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drinks whose popularity has been increasing since 1990s have also contributed to an elevation of caffeine intake, especially among teenagers and adolescents. [6] The intake of caffeine in high amounts may have adverse effects on the body. [2, 7] Moreover, besides caffeine, energy beverages usually contain other ingredients, including vitamins, taurine, glucuronolactone, etc. Their combined action and influence of separate constituents on the body have not been fully elucidated.

Cytotoxic effects of energy drinks and their components have been demonstrated in several papers. [8-10] Regeneration of glial cells is known to be a compensatory reaction in response to injury and neuronal cell death. [11] Ki-67 is a widely recognized nuclear cell proliferation marker and its expression is associated with compensatory activation of cell proliferation in response to glia and neuronal cell death.[12] Major neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and β-nerve growth factor (β-NGF) have been shown to promote growth and differentiation of certain neurons and synapses in the central and peripheral nervous systems.[13,14] These proteins also provide neuroprotective effects against certain insults and decreased levels of BDNF have been reported in neurodegenerative diseases.[15-17] Features of regenerative changes as a result of brain tissue injury by caffeinated energy drinks have not been previously studied.

Thus our study objective is to investigate Ki-67 expression in the brain tissue and neurotrophic factors BDNF and β-NGF levels in brain homogenates in laboratory rats after 2 months of oral consumption of caffeinated energy drinks.

MATERIALS AND METHODS

Description of animals and groups

Three-month-old female WAG rats weighing 180-200 g were provided by the vivarium of Kharkiv National Medical University. Twenty rats were randomly divided into two groups: experimental (n=10) and control (n=10). Animals from the experimental group orally consumed 'Black' caffeine energy drink (12 ml/kg) containing 320 mg/l of caffeine, guarana extract, taurine, and group B vitamins for two months on a daily basis (except weekends). The control group included animals administered drinking water instead of energy drinks.

Bioethics

All experimental procedures were approved by the Committee of Ethics and Bioethics at Kharkiv National Medical University. We strictly followed the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and Directive 2010/63/EU on the protection of animals used for scientific purposes adopted on September 22, 2010.

Immunohistochemical study of cell proliferation markers

To assess the rate of proliferation of glial and other cells, we chose a well-characterized proliferation marker Ki-67. It is a nuclear protein expressed in dividing cells only.[12]

As soon as animals were sacrificed strictly 2 months after the beginning of experiment, the brain was isolated and its fragments were fixed in 10% formalin solution. After incubation, formalin-fixed paraffin-embedded tissue blocks were prepared. Then 4-μm-thick sections were obtained from the paraffin-embedded brain tissues using a microtome. The sections were immunostained with mouse monoclonal antibodies to Ki-67 from *Thermo Fischer Scientific* (UK). After incubation with the primary antibodies to Ki-67, the sections were treated with an anti-(mouse IgG)–horseradish peroxidase conjugate. 3,3'-Diaminobenzidine (DAB) was used for visualization. For histological assessment, 4-μm-thick sections were obtained and stained with Einarson's gallocyanin-chrome

alum staining.

Preparation of brain tissue homogenates

To determine the content of BDNF and β-NGF, the brain was used to prepare homogenates. It was extracted and placed on ice. Then it was weighed and cut into small pieces. A Potter homogenizer was used to prepare homogenates in an isolation medium (1:10 proportion) during 30 seconds on ice with a glass-teflon gap of 0.2 mm. The isolation medium was composed of 0.25 M sucrose, 0.2 M Tris-HCl, and 1 mM EDTA (pH 7.4). After homogenization the suspension was centrifuged at 3,000 rpm during 10 min. The supernatant obtained as a result was used for quantitative determination of the neurotrophic factors mentioned above.

Determination of levels of neurotrophic factors BDNF and β-NGF in cerebral homogenates

We quantitatively determined levels of BDNF and β-NGF in brain homogenates of animals from both experimental and control groups. To measure BDNF concentrations, we used ELISA kits from *R&D Systems* (Minneapolis, USA). The content of β-NGF was also measured by ELISA using kits developed by *RayBiotech* (Norcross, USA). The corresponding assays were carried out in accordance with manufacturer's instructions in 96-well plates. After the addition of Stop solution, absorbance was measured at once at 450 nm using an Awareness Technology Stat Fax 303 Plus Microstrip Reader (USA). Levels of both BDNF and β-NGF were expressed in pg/ml.

Statistics

We used a "GraphPad Prism 5" application for performing all statistical evaluations. Mann-Whitney U test was used to compare two independent groups of variables. The results were considered statistically significant at $p < 0.05$.

RESULTS

Histological slides stained with Einarson's gallocyanin-chrome alum staining revealed that the brain tissue in rats from the control group was well preserved. Ventricular ependyma and epithelial cells of the choroid plexus were intact. Glial cells were not seen accumulating around the third ventricle. Rats from the experimental group had histological features of edema (Figure 1a) in the brain and were noted to have a periventricular zone of young non-differentiated glial-like cells, which are mainly observed during fetal and early neonatal periods. In addition, neuropil of various brain regions in rats from the experimental group seemed to be less dense compared with the control group, suggesting a decrease in the number of processes both in neurons and macroglia.

Immunohistochemical staining using antibodies to Ki-67 revealed that the amount of Ki-67 positive cells in the brain parenchyma of control animals was low. Ki-67-expressing cells were virtually absent in ventricular ependyma, vascular endothelium, and epithelium of the choroid plexus. Only solitary Ki-67-positive gliocytes were observed (Fig. 1b). However, they were found not in each field of vision. In contrast to the experimental group, the zones of macroglia proliferation were not found in the control animals.

Experimental animals had increased number of Ki-67 labeled gliocytes distributed diffusely in the brain tissue (Fig. 2a, 2b, 2c, 2d). In addition, such cells were found in the periventricular region (Fig. 2a, 2c). Moreover, Ki-67 staining was observed in ventricular ependyma and endothelium of microcirculatory blood vessels (Fig. 2b).

Determination of neurotrophic factors in the brain tissue homogenates showed that the content of BDNF in the brain of experimental animals was significantly reduced compared to control animals ($p=0.02$). Levels

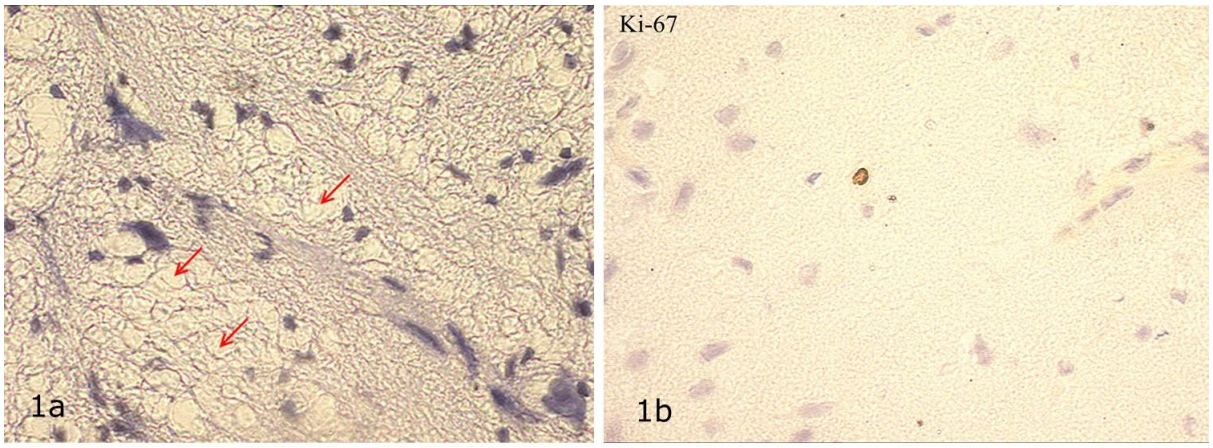

Figure 1: a) Brain of a rat from the experimental group. Signs of brain edema are marked with red arrows. Einarson's gallocyanin-chrome alum staining. x 400. b) Brain of a rat from the control group. Single Ki-67-labeled cell is visible. Immunohistochemical reaction with antibodies to Ki-67. x100.

peaked at 243.6 [IQR: 180.8, 351.9] pg/ml in the experimental group against 515.6 [IQR: 396.7-583.1] pg/ml in the control animals (Fig. 3). The difference between concentrations of β-NGF in brain homogenates was not statistically significant (p=0.33) with a trend towards its reduction. The levels of β-NGF were 11.78 [95% CI: 11.07-13.07] pg/ml compared to 12.41 [95% CI: 10.41-14.66] pg/ml in control animals (Fig. 4).

DISCUSSION

Histological observations (edema and low neuropil density, i.e. a decrease in the amount of unmyelinated axons, dendrites and glial cell processes) in animals from the experimental group suggested neurons and glial cells injury leading to cell death and reduction of neuronal and glial cell density, as a result of oral consumption of caffeinated energy drinks. Since neuropil is a synaptically dense area and cognitive functions such as learning and memory require plasticity based on the formation of new synaptic contacts that develop in the neuropil, low neuropil density observed in this study may be accompanied by a decrease in the amount of synapses and the reduced ability to form the new ones, negatively affecting the cognitive function. [18]

Zeidán-Chuliá et al have demonstrated that major ingredients of caffeinated energy drinks, including caffeine, taurine, and

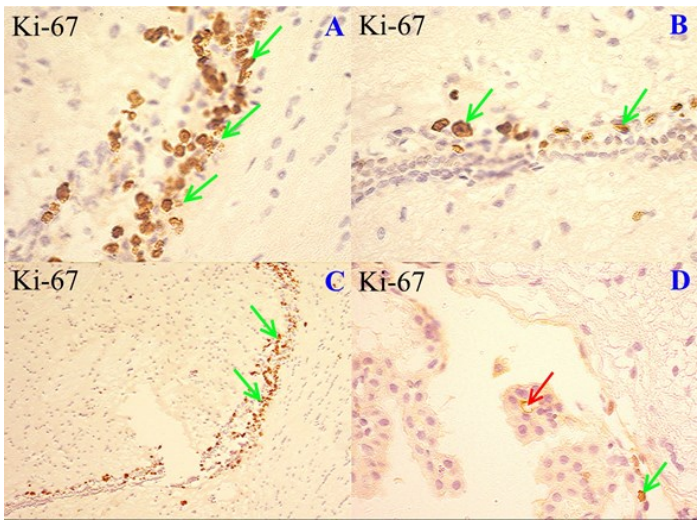

Figure 2: Brain Ki-67 immunostaining. A) A microslide of the third ventricle of an animal from the experimental group. Strong Ki-67 immunostaining is observed in ventricular ependyma and glial cells of the periventricular space (marked with green arrows). Immunohistochemical reaction with antibodies to Ki-67. x400. B) A fragment of the third ventricle of a rat from the experimental group. Ki-67-expressing gliocytes are marked with green arrows. Immunohistochemical reaction with antibodies to Ki-67. x400. C) A microslide of the third ventricle of a rat from the experimental group. Ki-67-positive ependymal cells and periventricular glial cells can be seen (marked with green arrows). Immunohistochemical reaction with antibodies to Ki-67. x100. D) A microslide of a lateral ventricle of the brain of a rat from the experimental group. Ki-67 is expressed in some ependymocytes, endothelial cells of capillaries (marked with a red arrow), and epithelial cells in the choroid plexus (marked with a green arrow). Immunohistochemical reaction with antibodies to Ki-67. x400.

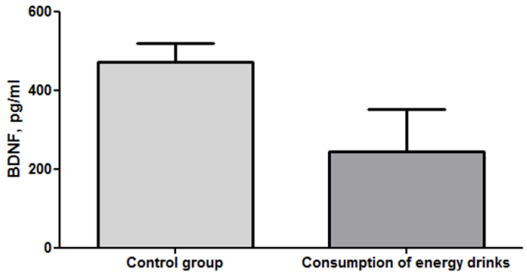

Figure 3: The content of BDNF in brain tissue homogenates of rats after the oral two-month-long consumption of caffeinated energy drinks is provided. Oral intake of caffeine-containing energy beverages resulted in a decrease in the amount of BDNF in the brain of animals (p=0.02).

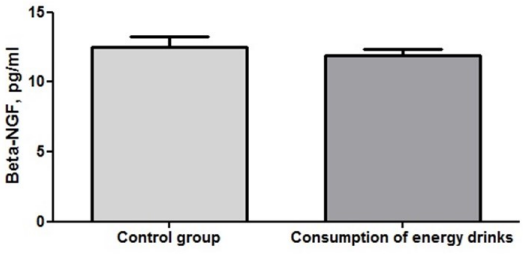

Figure 4: Levels of β-NGF in the brain tissue homogenates of rats after the two-month-long consumption of caffeinated energy drinks are shown. Statistically significant changes between two groups were not revealed (p=0.33).

guarana are cytotoxic to human neuronal cells. [10] It has also been reported that the consumption of caffeinated energy drinks against the background of alcohol intake promotes neuronal cells apoptosis in the hippocampus and temporal cortex of rats. [9] Bawazir AE showed that three-week-long oral consumption of caffeinated energy drinks at a dose of 3.1 ml/day resulted in cell degeneration and necrosis in the hippocampus. [8] Ki-67 upregulation in the brain indicates the activation of glia proliferation. Such changes in Ki-67 expression may be due to the increased requirements of glial cells as a result of neuronal cell death. [19] In addition, it is worth noting that Ki-67 overexpression in vascular endothelial cells, which is indicative of their proliferation probably due to the action of caffeinated energy drinks, may indicate their damage and contribute to subsequent higher nonselective permeability of blood-brain barrier composed of these cells, since damage to vascular endothelial cells that form the blood-brain barrier increases its permeability. [20] Our findings on the negative impact of caffeinated energy drinks on endothelial cells are consistent with data of other researchers. Worthley MI et al reported that the intake of caffeinated energy drinks by humans was accompanied by the decreased vascular endothelial function. [21] This may reduce the protective effects of the blood-brain barrier and facilitate the transport of constituents of caffeinated energy drinks to the brain.

Analysis of the content of neurotrophic factors BDNF and β-NGF in the brain tissue homogenates of experimental animals revealed that the prolonged oral intake of caffeinated energy drinks was associated with BDNF deficiency against the background of unaffected β-NGF levels. This suggest that the mechanism of brain injury caused by caffeinated energy drinks may involve reducing BDNF levels, hence reducing the neuroprotective effects as well as reducing neuronal growth and differentiation. BDNF is known to be implicated in learning, memory, and synaptic plasticity. [22] Given the low neuropil density described above and BDNF deficiency, we further hypothesize that chronic intake of caffeinated energy drinks affects cognitive functions and BDNF deficiency may play a significant role in realization of caffeinated energy drinks-associated cognitive dysfunctions. Moreover, Lipsky RH and Marini AM demonstrated that BDNF promoted survival of neurons and protected them from damage. [23] Thus, BDNF deficiency may contribute to and facilitate cell death of neurons and macroglia if consumption of caffeinated energy drinks is not stopped, making damage to the brain more and more severe.

Our findings that the long-term oral exposure to caffeinated energy drinks causes Ki67 overexpression and BDNF deficiency in the brain of rats confirmed previouos published reports on toxicity effects of caffeinated energy beverages. Translating these results

into clinical practice may require further clinical studies to confirm our hypothesis above regarding the mechanism of adverse effects of caffeinated energy drinks and their components on the nervous system (increased expression of Ki-67 and reduced BDNF levels) in clinical subjects.

CONCLUSION

Thus our study indicated that prolonged two months oral consumption of caffeinated energy drinks in rats was associated with histological evidence of neural injury (brain edema and reduced neuropil density) with overexpression of Ki-67 indicating regeneration of glial cells, ventricular ependymal cells and vascular endothelial cells in response to neuronal injury. In addition, this was observed against a background deficiency of brain-protective BDNF, observed as a result of prolonged consumption of high-energy caffeinated drinks. At present, the consumption of caffeinated energy drinks is increasingly popular among the young and working population, our findings may be clinically relevant to public health professionals in educating regular users of caffeinated energy drinks regarding the health hazards associated with their long-term consumption.

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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