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# LONG-TERM EXPOSURE TO CAFFEINATED ENERGY DRINKS PROMOTES APOPTOSIS OF CARDIOMYOCYTES AND RESULTS IN ENDOTHELIAL DYSFUNCTION IN MYOCARDIAL BLOOD VESSELS

Galina Gubina-Vakulyck[1], Anatolii Onishchenko[2], Oksana Nakonechna[2], Andrii Lupyr[3], Tatyana Gorbach[2], Alla Bondareva[2], Svetlana Stetsenko[2], Viktoriya Tkachenko[4], Hanna Polikarpova[2] and Anton Tkachenko[2*]

[1]*Department of Pathological Anatomy, Kharkiv National Medical University, Nauky ave 4, 61022, Kharkiv, Ukraine*
[2]*Department of Biochemistry, Kharkiv National Medical University, Nauky ave 4, 61022, Kharkiv, Ukraine*
[3]*Department of Otorhinolaryngology, Kharkiv National Medical University, Nauky ave 4, 61022, Kharkiv, Ukraine*
[4]*Department of Microbiology, Immunology and Virology, Kharkiv National Medical University, Nauky ave 4, 61022, Kharkiv, Ukraine*

*Corresponding Author: antontkachenko555@gmail.com

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**Abstract**

This study aimed to assess the apoptosis rate and endothelial function of the heart of rats that were orally exposed to a caffeinated energy drink (CED). The rats were orally exposed to CED for two weeks. The content of caspase-3, endothelial NO-synthase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor A (VEGFA), the activity of poly-(ADP-ribose) polymerase (PARP) was determined in the heart homogenates. Expression of heat shock protein 90α (HSP90α) and proliferation-associated Ki-67 protein in the heart were evaluated. Biochemical analysis demonstrated the activation of apoptosis in the heart of rats exposed to CED with the evidence of the elevated concentration of caspase-3 and the reduced activity of PARP. Consumption of CED was accompanied by the development of endothelial dysfunction in the cardiac vasculature, which was confirmed by a higher content of ET-1 and VEGFA in heart homogenates and HSP90α overexpression in endothelial and endocardial cells. Oral exposure to CED for two weeks is associated with enhanced apoptosis of cardiomyocytes and the development of endothelial dysfunction in the cardiac blood vessels.

## INTRODUCTION

Caffeinated energy drinks (CEDs) are non-alcoholic beverages that contain caffeine as a major ingredient and may have other methylxanthines, taurine, γ-gluconolactone, group B vitamins, guarana extract, carnitine, and high content of sugar. They successfully entered the European market in 1987 and have been gaining popularity since that time. Manufacturers of CEDs claim that their products can improve mental and physical productivity, performance, and endurance [1-3]. According to some estimates, almost one-third of adolescents in the United States daily consume CEDs [4]. Components of energy drinks have been reported to have some adverse health effects, in particular, on the cardiovascular system.

Caffeine is the most studied ingredient of CEDs and its impact on the cardiovascular system is well documented. It increases the heart rate and blood pressure, promotes the release of stress-related hormones [5]. However, Turnbull et al. indicate that such effects are transient and reversible and the moderate caffeine consumption (up to 600 mg/day) does not lead to the increased risks of cardiovascular pathology [6]. Taurine has been reported to exert cytoprotective effects in the heart and vessels due to its antioxidant properties, beneficial effects on energy metabolism, anti-hypertensive and anti-atherogenic activities [7, 8]. Glucuronolactone is

another ingredient of CEDs. This glucose derivative has also been demonstrated to provide favourable health effects: detoxification and platelet anti-aggregation [9]. CEDs contain high doses of B vitamins that usually exceed their recommended daily allowance. There is strong evidence that these vitamins decrease the risk of mortality from some cardiovascular diseases and reduce the circulating level of homocysteine [9, 10]. Despite the reported beneficial effects of individual components of CEDs on the cardiovascular system, the consumption of energy drinks, i.e. the combined action of all constituents, is associated with a negative impact on the cardiovascular system [11-13].

Cell death and survival are fundamental for the maintenance of tissue homeostasis, including in the heart. One of the cell death modes called apoptosis, which is a programmed self-destruction, is involved in the regulation of homeostasis in the heart. Both extrinsic and intrinsic pro-apoptotic signals finally result in the activation of proteolytic enzymes caspases whose function is to degrade intracellular proteins promoting cell death [15]. Apoptosis of cardiac myocytes may lead to their loss and organ dysfunction, especially given that the heart has a very limited regenerative capacity [15, 16].

The endothelium is a monolayer of cells that cover the vessels from inside with numerous functions, including regulation of vascular tone, coagulation, angiogenesis, inflammation, etc [17]. Its proper function in the coronary vessels is of vital importance for the heart and endothelial dysfunction is known to be associated with abnormal coronary perfusion and, thus, decreased exercise capacity [18]. Endothelial dysfunction develops if there is an imbalance between vasoconstrictors such as some arachidonic acid derivatives, endothelin-1 (ET-1), angiotensin II and vasodilators such as NO produced by endothelial NO-synthase (eNOS), prostacyclins, etc. [19]. Another important agent involved in the regulation of endothelial function and promotion of angiogenesis, which is crucial for normal tissue development and repair, is vascular endothelial growth factor-A (VEGFA) [20]. Thus, we want to test the hypothesis that oral exposure to CEDs by laboratory animals may negatively affect the endothelial function and promote apoptosis of cardiomyocytes.

The aim of our study was to evaluate the detrimental effects of oral exposure to CEDs on the heart by assessing apoptosis and endothelial markers as well as expression of stress-induced heat shock protein 90α (HSP90α) and proliferation-associated Ki 67 in rats.

## MATERIALS AND METHODS

### Study Design

Adult female WAG rats weighing 160-190 g were housed in the vivarium of Kharkiv National Medical University. Females are more sensitive to the adverse effects of caffeine-containing beverages, which explains why rats of this gender were selected for this study [21]. The animals were housed in cages in standard laboratory conditions at room temperature (24 ± 2 °C) and relative humidity of 50-60 %. *Food* was provided *ad libitum*. Sixteen rats were randomly subdivided into two equal groups: experimental (n=8) and control (n=8). The animals from the experimental group were orally administered a caffeinated energy drink (12 ml/kg) for two weeks daily (except for weekends) via an oral gavage using a plastic tube. The non-alcoholic, carbonated energy drink "Black" (LLC TC GOOD FOOD, Cherkasy, Ukraine) was used. It contained 320 mg/L of caffeine, taurine, vitamins B3, B5, B6, B12, guarana extract, inositol, citric acid, trisodium citrate, sodium benzoate, potassium sorbate, sugar, and inverted sugar syrup. The control group included animals administered drinking water instead of energy drinks.

All institutional and national guidelines for the care and use of laboratory animals were strictly followed. The study was approved by the Commission of Bioethics at Kharkiv National Medical University (Kharkiv, Ukraine).

The experiment was designed and all manipulations with animals were performed according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

### Preparation of Myocardium Homogenate

The rats were anaesthetized and sacrificed. The heart was immediately excised and washed with ice-cold phosphate-buffered saline (PBS; Becton Dickinson, San Diego, CA, USA). Then 300 mg of cardiac tissue was collected on ice and sliced into small sections using scissors. Slices were homogenized in a PBS-based homogenization buffer containing 0.25 m sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ (reagents were purchased from KharkivReaChim, Kharkiv, Ukraine). Homogenates were centrifuged at 12000g for 20 minutes at 4 °C. The supernatants were apportioned into 1 ml aliquots and stored at –51 °C until ready for determination of the poly-(ADP-ribose) polymerase (PARP) activity and caspase-3 levels.

### Determination of Apoptosis, Endothelial Dysfunction and Angiogenesis Markers in Myocardium Homogenates

The activity of poly-(ADP-ribose) polymerase (PARP) in myocardium homogenates was measured [22] based on the determination of poly-ADP-ribose after the electrophoretic separation of nuclear poly-ADP-ribosylated proteins. The activity of PARP is expressed in μmol of poly-ADP-ribose/mg protein.

The content of caspase-3, VEGFA, eNOS and ET-1 in myocardium homogenates was determined using ELISA kits

in accordance with the guidelines provided by the manufacturers (Rat CASP3 (Caspase 3) ELISA Kit; Rat VEGF-A (Vascular Endothelial Cell Growth Factor A) ELISA Kit; Rat NOS3/eNOS (Nitric Oxide Synthase 3, Endothelial) ELISA Kit; Rat ET-1 (Endothelin 1) ELISA Kit, respectively). All kits were purchased from Elabscience (USA). Numerical values of the optical density of solutions were registered using the Awareness Technology Stat Fax 303 Plus Microstrip Reader (USA). The content of all parameters determined by ELISA was expressed in pg/mg protein. Protein levels in myocardium homogenates were quantitatively determined using the Lowry assay [23].

**Immunostaining for Ki67 and HSP90α**

Ki-67 and HSP90α expression was immunohistochemically detected in samples of the myocardium in rats from both control and experimental groups. The tissue samples were collected when the animals were sacrificed. They were fixed in a 10% neutral formalin solution. Paraffin-embedded tissues were used to prepare 4 μm-thick sections, which were stained with the first anti-Ki-67 and anti-HSP90α antibodies purchased from "Thermo Fischer Scientific" (UK) and then the second horseradish peroxidase-conjugated antibodies were used. 3,3′-Diaminobenzidine (DAB) staining was used for visualization. Axiostar-plus (Zeiss) microscope was used for the analysis of results.

**Statistical Analysis**

Experimental data were processed using GraphPad Prism 5 software. Non-parametric Mann-Whitney U test was selected to compare the numerical values of two independent groups. Differences between groups were considered statistically significant at $p < 0.05$.

## RESULTS

Oral exposure of CEDs by laboratory animals was found to affect both apoptosis biomarkers studied in this research. In particular, the content of caspase-3 in myocardium homogenates was statistically significantly 57% higher in the experimental group compared with the controls (Table 1). Analysis of the activity of PARP revealed that it statistically significantly decreased as a result of CED consumption. The activity of PARP was twice as low in rats orally exposed to energy beverages in comparison with the control group (Table 1).

**Table 1.** Parameters of apoptosis in myocardial homogenates of rats orally exposed to caffeinated energy drinks (Me [IQR])

| Parameter | Control group | Rats exposed to caffeinated energy drinks |
|---|---|---|
| Concentration of active caspase -3 (pg/mg protein) | 21.85 [21.45; 23.96] | 34.26 [33.05; 36.97] $p < 0.001$ |
| Activity of poly (ADP-ribose) polymerase (PARP) (μμmol of poly-ADP-ribose/mg protein) | 1.59 [1.47; 1.74] | 0.79 [0.70; 0.92] $p < 0.001$ |

**Note:** a p-value less than 0.05 (typically ≤ 0.05) is statistically significant

When comparing the content of endothelial function markers, we found no statistically significant changes in the level of eNOS in myocardium homogenates between both groups (Table 2). However, the content of ET-1 was markedly and statistically significantly 123% higher in rats administered CEDs in myocardium homogenates than in controls (Table 2). Furthermore, we determined the concentration of VEGFA in myocardium homogenates of animals exposed to energy drinks. It was found to be 33% elevated in the experimental group compared with intact animals. The changes were statistically significant (Table 2).

The myocardium in animals from the control group was intact with no signs of damage to cardiomyocytes. $Ki67^+$ cells were found in small amounts both in the stroma and in the intima of vessels (Fig. 1). The expression of Ki67 was limited to the nucleus. It is important to note that the amount of $Ki67^+$ cells was found to be higher in the stroma in the experimental group compared to controls. Furthermore, the amount of dividing Ki67-expressing cells in the endothelial lining of blood vessels of various diameters was higher than in the control group (Fig. 2).

**Table 2.** Parameters that characterize the state of vascular endothelium in myocardial homogenates of rats orally exposed to caffeinated energy drinks (Me [IQR])

| Parameter | Control group | Rats exposed to caffeinated energy drinks |
|---|---|---|
| Endothelial NO-synthase (pg/mg protein) | 19.23 [18.95; 21.76] | 20.79 [19.97; 22.12] $p > 0.05$ |
| Endothelin-1 (pg/mg protein) | 1.47 [1.23; 1.58] | 3.28 [2.98; 3.84] $p < 0.001$ |
| VEGFA (pg/mg protein) | 121.5 [113.0; 135.2] | 163.1 [144.2; 185.0] $p < 0.05$ |

**Note:** a p-value less than 0.05 (typically ≤ 0.05) is statistically significant.

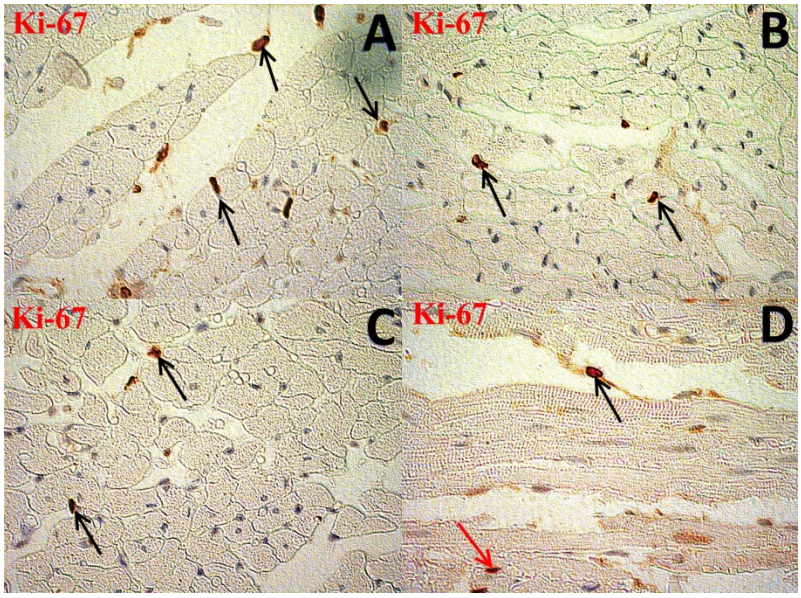


**Figure 1.** Immunostaining for nuclear proliferation marker Ki-67 in the heart of a rat from the control group. Ki-67$^+$ cells are observed in small amounts in vascular endothelia and endocardium. Nuclear expression of Ki-67 is marked using arrows. 400x.

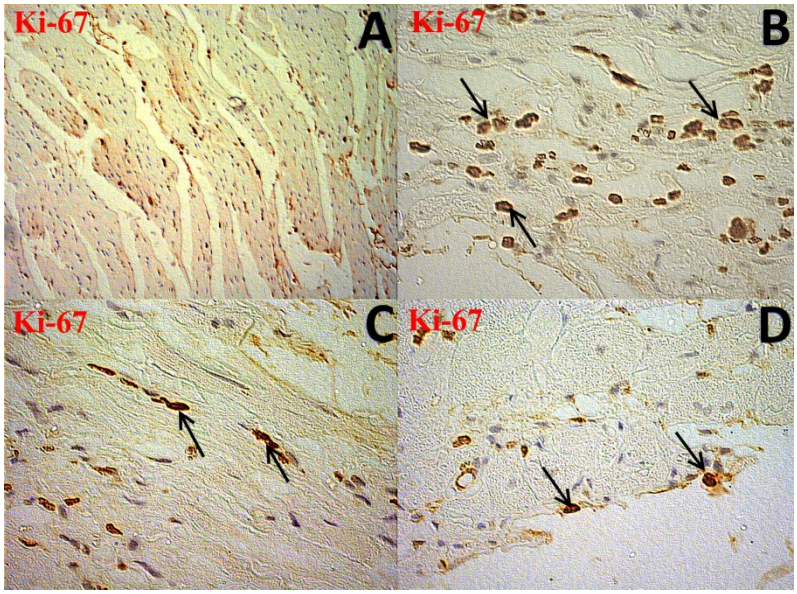


**Figure 2.** Immunostaining for Ki-67 in the heart of rats consumed a caffeinated energy drink during 2 weeks. A higher amount of Ki-67-labelled cells are found both in the endothelial and endocardial linings. Furthermore, the focal overexpression of proliferating cells can be revealed in the stroma. Nuclear expression of Ki-67 is shown with arrows. Figure 2a: 100x; Figures 2b, 2c, 2d: 400x.

Immunostaining for HSP90α in the myocardium of intact animals revealed a low level of its expression. Expression of the chaperone was detected in both endothelium and cardiac myocytes. However, some foci of stronger expression could be found against the background of weak diffuse immunostaining (Fig. 3). Stronger diffuse expression of HSP90α was revealed in the myocardium of rats from the experimental group. Against the background of strong diffuse staining, small foci with even more pronounced expression were observed (Fig. 4). It is worth mentioning that HSP90α was significantly upregulated in endocardiocytes.

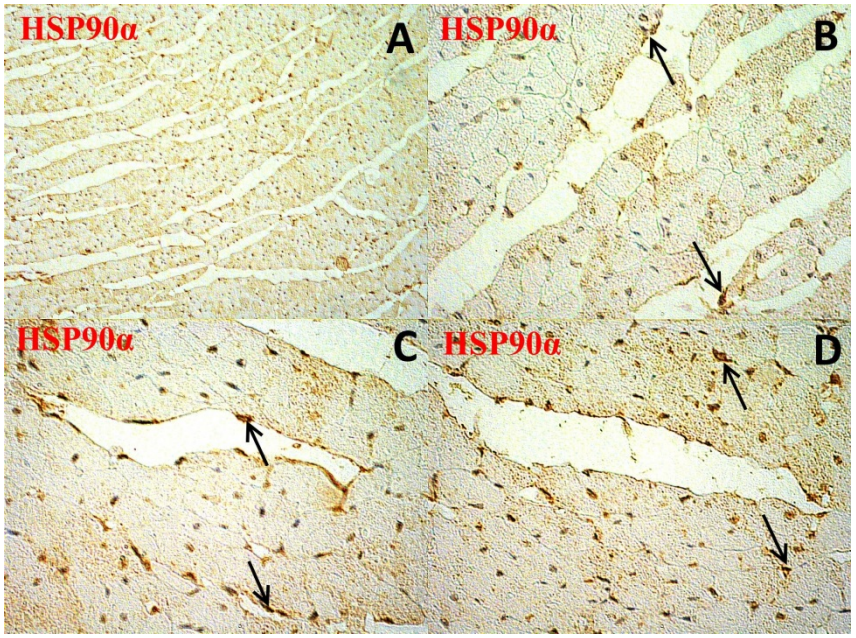


**Figure 3.** Immunostaining for HSP90α in the heart of a rat from the control group. A) Diffuse brown staining indicates weak expression of HSP90α. 100x. B, C, D). HSP90α expression can be observed in cardiac myocytes and endothelial cells. Foci of HSP90α overexpression are marked with arrows. 400x.

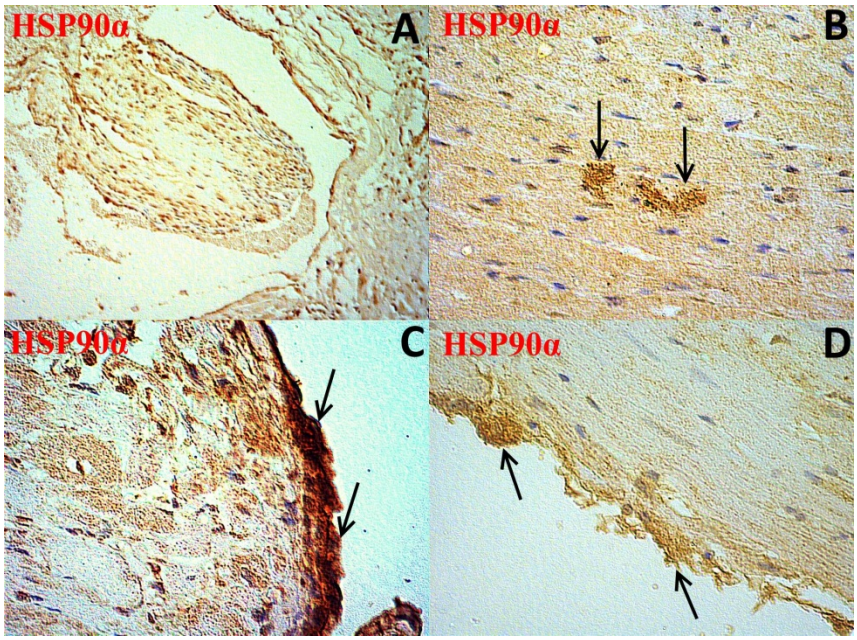


**Figure 4.** Immunostaining for HSP90α in the heart of rats orally exposed to a caffeinated energy drink. Diffuse HSP90α staining is stronger than in the control group (A; B). A strong expression of HSP90α is found in a papillary muscle, endocardium and surrounding myocardium (A). A region with the strongest HSP90α expression of irregular shape, shown using arrows, can be noticed in the myocardium, presumably it is a focus of micronecrosis (B). Such foci were not observed in the control group. Endocardial cells are characterized by an extremely strong expression of HSP90α, which is marked with arrows (C; D), indicating damage to these cells. Figure 4a: 100x; Figures 4b, 4c, 4d: 400x.

## DISCUSSION

Apoptosis is known to be one of the numerous cell death modes, which is characterized by shrinkage of cells, their fragmentation into apoptotic bodies and subsequent engulfment of them by phagocytic cells. Cleavage and activation of caspase-3 is a key step of apoptosis since this proteolytic enzyme is responsible for the degradation of the majority of proteins in apoptosis making active caspase-3 a reliable marker of apoptosis [24]. Another enzyme whose

cleavage is observed during apoptosis is PARP. Under normal circumstances, this enzyme is involved in DNA repair. In apoptosis, PARP is degraded under the influence of various proteases, including caspase-3, and its degradation is considered a hallmark of apoptosis [25, 26]. Thus, our findings of the reduced activity of PARP and activation of caspase-3 in myocardium homogenates indicate an intensification of cardiac myocyte apoptosis in response to CED intake. Moreover, foci of HSP90α overexpression found in this study in rats from the experimental group may indicate micronecrosis of cardiac tissue with dying or dead cardiomyocytes. Such a conclusion adheres to the findings of Slawinski et al. [27]. According to that study, consumption of CED during eight weeks *ad libitum* resulted in induced apoptosis of cardiomyocytes in rats. In this study, we showed that apoptosis could be induced by much shorter periods (2 weeks) of CEDs even at lower doses.

Munteanu et al. demonstrated that CEDs, especially in combination with alcohol, promote biochemical changes in the cardiac muscle such as accumulation of glycogen granules and reduced cholesterol content in the heart, which may cause myocardium dysfunction [28].

The intensified apoptosis in the heart may have a negative impact on its function. It has been reported that apoptosis of cardiomyocytes, which are the most abundant cells in the heart that provide contraction, is associated with some cardiovascular diseases, including ischemic heart disease, cardiac insufficiency, and cardiomyopathy [15]. It is believed that the reduced number of cells that are capable of contracting may lead to an increased workload on the remaining viable cells, which may result in depressing ventricular functions [29]. Thus, our findings and previous studies of other authors allow us to presume that the chronic consumption of CEDs may lead to cardiac dysfunction, including due to the loss of cardiomyocytes as a result of their apoptosis.

Cardiac myocytes are not divided to provide cardiac regeneration after lesions. Thus, cardiac repair occurs via the production of extracellular matrix by fibroblasts and the replacement of dead cardiac myocytes with a collagen-containing connective tissue [30]. A higher amount of $Ki67^+$ fibroblasts in the heart of animals from the experimental group may be due to excessive apoptosis of cardiac myocytes in response to consumption of CED. In addition, it was found that the damage to the heart caused by the consumption of CEDs was also medicated by the destruction of endothelia and endocardial lining.

## CONCLUSION

Consumption of CED during two weeks by rats causes activation of apoptotic processes in the heart and the development of endothelial dysfunction in cardiac vessels.

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## DISCLOSURE

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

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