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EXPERIMENTAL STUDY OF ERYTHROCYTE MEMBRANES IN RATS ORALLY EXPOSED TO CAFFEINATED ENERGY DRINKS BY FLUORESCENT PROBE TECHNIQUE

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Abstract: The aim of our research was to assess the state of red blood cell (RBC) membranes in rats orally administered caffeinated energy drinks during two months using the fluorescent probe O1O **(**2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole). Fluorescent probe O1O (2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole), which locates in the area of glycerol backbones, carbonyl groups of phospholipids, and hydrocarbon chains of phospholipids (near carbonyl groups) in the bilayer, was used to assess the state of RBC membranes in suspensions prepared from whole blood samples obtained from ten female adult WAG rats orally administered a caffeinated energy drink at a dose of 12 ml/kg during two months and ten controls. Energy drink consumption was associated with a higher fluorescence intensity of the phototautomer form of the probe O1O (the fluorescence maximum is at a wavelength of 480 nm) in erythrocyte suspensions compared with the control animals. The observed change in the fluorescence of the probe is attributed to the increase in the viscosity of the probe environment in the membrane. It was revealed using the fluorescent probe O1O that the long-term oral administration of caffeinated energy drinks to rats caused the increased membrane viscosity (i.e. reduced fluidity) in RBCs.

Keywords: caffeinated energy drinks, fluorescent probe, rats, cell membrane.

INTRODUCTION

Caffeinated energy drinks (CEDs) have become extremely popular among adolescents and young adults worldwide. They are believed to improve physical performance, endurance, and cognitive processes (Richards and Smith, 2016; Alsunni 2015). The effects of energy drinks on mental and physical performance are mediated by the high amount of caffeine. Some energy drinks may contain up to 550 mg per a can or a small bottle, whereas the caffeine toxicity threshold is considered to be 400 mg/day for adults (Temple et al., 2017; Richards and Smith, 2016). Taking into account the fact that CEDs are primarily consumed by teenagers for whom the caffeine daily intake should be limited to 100 mg only, the problem of their consumption by children and adolescents seems to be of huge importance. In addition to caffeine, the list of energy drink constituents includes: glucuronolactone, taurine, L-carnitine, group B vitamins, etc. (Richards and Smith, 2016; Alsunni 2015).

The impact of CEDs as a whole and their ingredients in particular on the health of laboratory animals has been actively studied. It has been reported about numerous adverse effects of energy drinks (Richards and Smith, 2016; Alsunni 2015). However, there is no information on the influence of energy drink intake on the state of erythrocyte membranes.

The aim of our research was to study the state of erythrocyte membranes in rats after the two-month-long oral intake of CEDs using fluorescent probe O1O **(**2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole**).**

MATERIALS AND METHODS

Description of laboratory animals used in this study and groups

According to the conditions of our experiment, twenty female adult WAG rats weighing 180-200 g were randomly divided into control and experimental groups. The rats from the experimental group were administered a CED of a famous brand daily (except for weekends) once at a dose of 12 ml/kg of body weight for 60 days (n = 10). The control group consisted of 10 intact animals.

Preparation of RBC suspensions and description of the fluorescent probe

The suspension of erythrocytes was obtained from blood of animals using saline physiological solution. To prepare RBC suspension for incubation with the fluorescent probes, RBCs were washed. Briefly, when the rats were sacrificed, their whole blood was collected into sterile sodium citrate VACUTAINER blood collection tubes. Then 100 μm of blood was added to 12 x 75mm capped polysterene test tubes containing 1 ml of normal saline solution (0.9% NaCl, Yuria-Pharm, Ukraine). This was followed by centrifugation at 2000 rpm during 5 minutes. The supernatant was discarded. RBCs were resuspended in 1% of normal saline solution and centrifuged again in the same conditions. This step was repeated to have a total of 3 washes. Then 10 μm of erythrocyte mass was added to 1 ml of 0.9% NaCl solution. The suspension obtained as a result was at once used for the incubation with the fluorescent probe. The fluorescent probe O1O

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was dissolved in acetonitrile to the initial concentration of $2 \cdot 10^{-4}$ mol/L. Fifty microliters of the probe solution was added to the RBC suspensions. The final content of the probe in RBC suspensions reached $5 \cdot 10^{-6}$ mol/L. The lipid-to-probe molar ratio was 200:1. The fluorescence of probe O1O (2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole) was measured in RBC suspensions of both groups using fluorescence spectrometer "Lumina" (Thermo Fisher Scientific). The measurement was carried out after 1 hour after the addition of the probe to RBC suspensions. The fluorescence spectra of probes were measured in the range of 360-600 nm with monochromator slit width of excitation and fluorescence 5 and 5 nm, respectively, and the excitation wavelength of 330 nm. The fluorescent probe O1O was chosen for our research, since its fluorescent characteristics depend on the physico-chemical properties of its microenvironment: the proton-donor ability, the polarity and viscosity of the microenvironment (Posokhov et al., 2018; Doroshenko et al., 2002; Doroshenko et al., 2000; Doroshenko and Posokhov 1999; Doroshenko et al., 1997).

It is well-known that the excited state proton transfer (ESIPT) reaction occurs when the ortho-hydroxy 2,5-diaryl-1,3-oxazole is in the excited state (Posokhov et al., 2018; Doroshenko et al., 2002; Doroshenko et al., 2000; Doroshenko and Posokhov, 1999; Doroshenko et al., 1997): hydroxyl group in the ortho-position of the lateral benzene ring acts as protonodonor, whereas the nitrogen atom of oxazole ring acts as proton acceptor (Figure 1). As a result of this reaction, phototautomer form (T*) is formed. It is fluorescent in significantly longer wavelengths compared with the initial (or so-called "normal") form (N*) (Posokhov et al., 2018; Doroshenko et al., 2002; Doroshenko et al., 2000; Doroshenko and Posokhov, 1999; Doroshenko et al., 1997): the fluorescence maximum of the phototautomer form of O1O is at 480nm, while the corresponding maximum for the normal form is at 375 nm.

The presence of two-band fluorescence provides us with the opportunity to perform ratiometric measurement, i.e. to use the ratio of the phototautomer form and the initial form fluorescence intensities (I_{T*}/I_{N*}) as a parameter for assessment of the physical and chemical properties of the microenvironment.

Usage of ratiometric fluorescent probes eliminates not only the measurement error caused by the deviation of the fluorescent probe concentration (e.g., uneven content of fluorescent probe in various membranes), but also the measurement errors due to deviation in configuration and adjustment of equipment for measurements of fluorescence (e.g., deviation in the intensity of the source of excitation light, changes in focusing, changes in the sensitivity of the photodetector, etc.) (Mély and Duportail, 2013).

Location and orientation of the probe 2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole (probe O1O) in the cell membrane is demonstrated in Figure 2. The probe O1O locates in the area of glycerol backbones of phospholipids (closer to the center of the lipid bilayer), in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids (near carbonyl groups of phospholipids).

The location and orientation of the probe O1O in the cell membrane are suggested on the base of its fluorescent properties in lipid membranes (Posokhov *et al*., 2018; Posokhov and Kyrychenko, 2018), calculations of its location using a method of molecular dynamics (Posokhov and Kyrychenko, 2018) and its structural similarity to the fluorescent probes with a known location and orientation in lipid membranes (Dobretsov, 1989).

Bioethics

The study was carried out 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). The experiment was approved by the Committee of Ethics and Bioethics of Kharkiv National Medical University.

Statistical analysis

Experimental data were processed using the "GraphPad Prism 5" software. Mann-Whitney U test was used to compare the numerical values of two independent groups. Data are represented as median and interquartile range. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

It was found that the oral consumption of energy drinks was accompanied by an increase in the fluorescence intensity of the phototautomer form of the probe (the emission maximum is at 480 nm) and, thus, by a higher value of the I_{480}/I_{375} ratio (Figure 3, Table 1). Taking into account rather low fluorescence intensity of the normal form (the emission maximum is at 375 nm), the mentioned changes in the fluorescence parameters of the probe point to the increase in the viscosity of the probe environment (Posokhov et al., 2018; Doroshenko and Posokhov, 1999).

Thus, fluorescent probe O1O allowed us to reveal that the prolonged oral administration of caffeine-containing energy beverages to rats is accompanied by the increased membrane viscosity (i.e. reduced fluidity) of RBCs.

Table 1.
Fluorescence intensity of probe O1O in RBC membranes of animals against the background of two-month-long consumption of energy drinks (median [interquantile 25%; 75%])

Groups of animals	Fluorescence intensity, a.u.		
	Probe O1O		
	375 nm	480 nm	I_{480}/I_{375}
Control (n=10)	16 [14; 19]	1276 [1201; 1338]	80 [72; 90]
Oral intake of caffeinated energy drinks (n=10)	18 [15; 20] p>0.05	2456 [2367; 2596] p<0.001	136 [120; 158] p<0.01

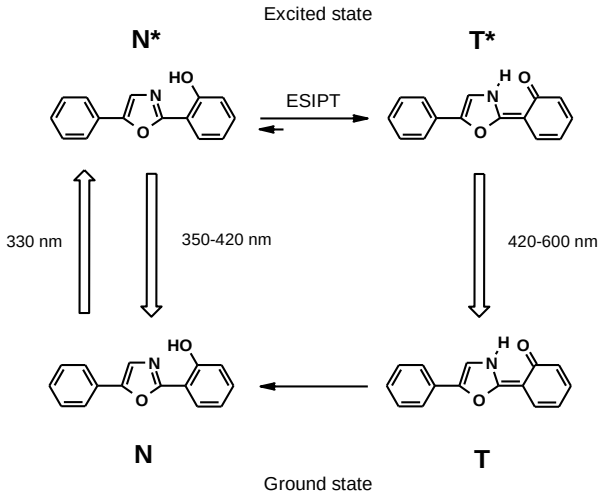

Fig. 1. Scheme of excited state intramolecular proton transfer (ESIPT) in 2-(2′-hydroxyphenyl) -5-phenyl-1,3-oxazole (probe O1O). The upwards arrow shows the electronic excitation and the downwards arrow represents the emission of light (fluorescence). Corresponding maximum of absorption and the ranges of emission are measured in nanometers.

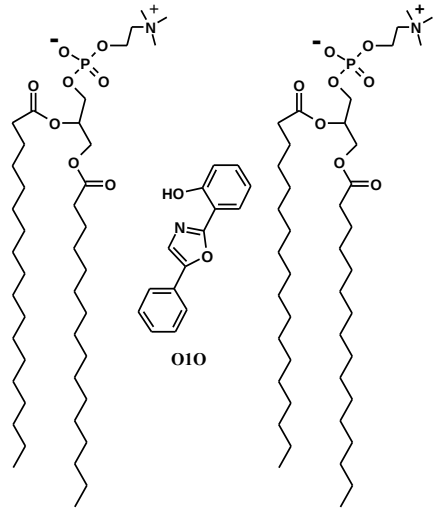

Fig. 2. Location and orientation of the fluorescent probe 2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole (probe O1O) in phospholipid membranes. Two molecules of phosphatidylcholine from the outer leaflet are shown to denote the location of the probe.

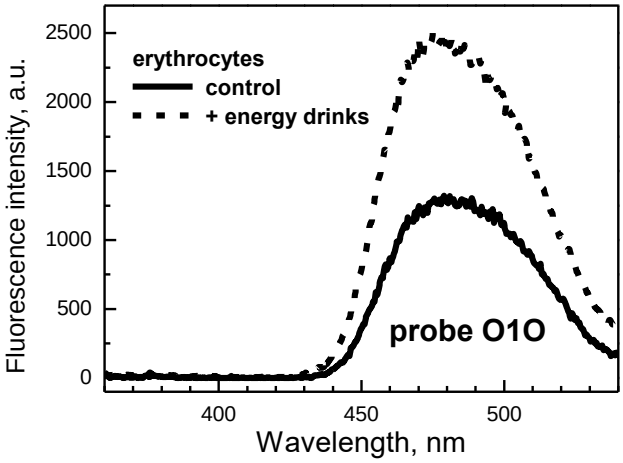

Fig. 3. The representative fluorescence spectra of the probe O1O in erythrocyte suspensions: (a) the control group (black solid line), (b) the animals exposed to energy drinks during two months (black dashed line).

DISCUSSION

Maintenance of membrane fluidity in RBCs is absolutely necessary for viability of these cells (Hollán, 1996). It provides the proper interaction of ligands with receptors embedded in cell membranes and affects the action of ion channels and transporters (Maulucci *et al*., 2016). Under normal conditions, the membrane fluidity and viscosity, which is the reciprocal of fluidity, depend on multiple factors, including structure of cell membranes and lipids involved in their formation, as well as on fatty acid composition of membrane-associated phospholipids, etc. (Maulucci *et al*., 2016). It has also been reported that lipid peroxidation and oxidative stress affect the membrane fluidity as a whole and of RBCs in particular, decreasing it and therefore increasing the membrane viscosity (Reiter et al., 2014; Tsuda 2010). Lipid peroxidation is primarily associated with the influence of free radicals on polyunsaturated fatty acids diminishing their amount in phopholipid bilayer and therefore increasing the relative percentage of saturated fatty acids, which results in the increased membrane viscosity and, thus, in the decreased membrane fluidity (Cazzola et al., 2004).

It is worth noting that the consumption of energy drinks results in the activation of lipid peroxidation processes and oxidative damage to liver and brain cells (Reis *et al*., 2017). Al-Basher GI et al reported that even perinatal exposure to CEDs induces lipid peroxidation and therefore promotes the development of oxidative stress (Al-Basher et al., 2018). Moreover, it is known that a higher rate of lipid peroxidation contributes to hemolysis due to the affected integrity of RBC membranes. Munteanu C et al demonstrated that the red blood cell count is reduced in rats orally administered CEDs. The authors attributed this decrease to the intensified destruction of RBCs (Munteanu et al., 2014). Thus, there is strong evidence that the oral long-tern consumption of CEDs is associated with the development of oxidative stress.

Our findings that suggest the association between the two-month-long consumption of CEDs and the increase in the viscosity of the lipid bilayer (i.e. with the decrease in the membrane fluidity), evidenced by the changes in the fluorescence of probe O1O, in RBCs of rats may be due to the activation of lipid peroxidation and the development of oxidative stress.

It is worth mentioning that the state of membrane affects oxygen diffusion capacity and thus respiratory function of RBCs (Cazzola et al., 2004). Thus, the energy drink consumption-associated increase in RBC membrane viscosity found in this study may affect oxygen transport efficiency and contribute to a decrease in tissue oxygenation and the development of hypoxia.

CONCLUSIONS

Our findings suggest that the long-term oral exposure of rats to CEDs result in an increase in viscosity of RBC membranes and, thus, leads to a decrease in fluidity of the membranes. One can suggest that the mentioned decrease in membrane fluidity, in its turn, potentially might affect the efficiency of gas exchange.

AUTHOR'S CONTRIBUTION

All authors contributed to planning and design of the study, data analysis, drafting and critically revising the paper. Each author mainly contributed in the following way: conceptualization: Yevgen Posokhov, Anton Tkachenko, Oksana Nakonechna; methodology: Yevgen Posokhov, Anton Tkachenko; data collection: Anatolii Onishchenko, Yevgen Korniyenko, Maryna Tkachenko; data validation: Anatolii Onishchenko; data processing: Yevgen Posokhov, Anatolii Onishchenko, Anton Tkachenko; writing-original draft

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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