Food and Chemical Toxicology 62 (2013) 343–348

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

No harmful effect of different Coca-cola beverages after 6 months of intake on rat testes

Ľubomíra Tóthová [a,*], Július Hodosy [a,b], Kathryn Mettenburg [c], Helena Fábryová [a], Alexandra Wagnerová [a], Janka Bábíčková [a], Monika Okuliarová [d], Michal Zeman [d], Peter Celec [a,e,f]

[a] Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia
[b] Institute of Physiology, Comenius University, Bratislava, Slovakia
[c] Iowa State University, Ames, IA, USA
[d] Department of Animal Physiology and Ethology, Comenius University, Bratislava, Slovakia
[e] Institute of Pathophysiology, Comenius University, Bratislava, Slovakia
[f] Department of Molecular Biology, Comenius University, Bratislava, Slovakia

ARTICLE INFO

Article history:
Received 9 May 2013
Accepted 26 August 2013
Available online 31 August 2013

Keywords:
Soft drinks
Coca-cola
Testes
Oxidative stress

ABSTRACT

Our laboratory recently reported that a 3-month exposure of rats to cola-like beverages induced sex hormone changes. The aim of the study was to investigate the effects of various types of Coca-cola intake with different composition for 6 months on oxidative status in testes and testosterone in adult male rats. Fifty adult male Wistar rats were divided into control group drinking water, and groups drinking different Coca-cola beverages (regular Coca-cola, Coca-cola caffeine-free, Coca-cola Light and Coca-cola Zero). Oxidative and carbonyl stress markers were measured in the testicular tissue to assess oxidative status together with testicular and plasma testosterone. StAR expression in testes as a marker of steroidogenesis was quantified. No significant differences were found between the groups in any of the measured parameters.

In conclusion, oxidative and carbonyl stress in testicular tissue were not influenced by drinking any type of Coca-cola. Additionally, testosterone in testes and in plasma, as well as testicular StAR expression were comparable among the groups.

1. Introduction

The worldwide consumption of sugar-sweetened beverages is very high (Ebbeling et al., 2012). According to observational studies, intake of sugar-sweetened beverages is associated with metabolic diseases such as metabolic syndrome, obesity, kidney diseases, and others (de Ruyter et al., 2012; Sheludiakova et al., 2012). Based on the observed associations authorities try to regulate intake of sugar-sweetened beverages, especially in adolescents. However, there is a lack of experiments supporting these associations and proving their causality (Farley et al., 2012), some even show the opposite (Celec, 2012).

The exact composition of Coca-cola beverages is confidential. The main components are nevertheless known: apart from water, it contains phosphoric acid, glucose/fructose or artificial sweeteners and caffeine. Phosphoric acid is present in all available Coca-cola beverages. Differences between various types of Coca-cola are mainly in sweetener and caffeine content (Table 1). The concentration of glucose/fructose in regular Coca-cola is relatively high. In Coca-cola caffeine-free, Light and Zero, sugar is substituted by artificial sweeteners such as acesulfame K, aspartame and cyclamate (Maes et al., 2012). Chronic high glucose/fructose intake may cause insulin resistance and subsequently high blood sugar concentration potentially leading to glucotoxicity (Solomon et al., 2012). High blood glucose reacts with proteins (as well as with oxidized lipids, bases in DNA and with other carbonyl substances) in a nonenzymatic Maillard reaction forming Amadori products and later compounds termed advanced glycation endproducts (AGEs) (Klenovics et al., 2013). Low molecular weight AGEs are mostly secreted to urine or bile, but high molecular weight AGEs tend to accumulate and trigger an immune response accompanied by increased production of reactive oxygen species (ROS) (Qin et al., 2013). A certain level of ROS is needed for several physiological processes in reproduction, such as acrosome reaction or hyperactivation and normal sperm function (de Lamirande and Gagnon, 1995). Overproduction of ROS, on the other hand, causes oxidative stress and damage of macromolecules: lipid peroxidation, protein oxidation and oxidation of DNA (Aitken and Roman, 2008).

Another compound found in regular Coca-cola is caffeine. Caffeine has been shown to have protective effects in liver, brain, epididymis and kidneys via prevention of lipid peroxidation and/or

* Corresponding author. Address: Institute of Molecular Biomedicine, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovakia. Tel.: +421 2 59357371; fax: +421 259357631.
E-mail address: tothova.lubomira@gmail.com (Ľ. Tóthová).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.08.073

Table 1
Review of main differences between individual types of Coca-cola beverages.

Component	Tap water	Coca-cola regular	Coca-cola Light	Coca-cola Zero	Coca-cola caffeine-free
Water	+	+	+	+	+
Glucose/fructose	–	+	–	–	–
Caffeine	–	+	+	+	–
Sodium cyclamate	–	–	+	+	+
Acesulfam-K	–	–	+	+	+
Aspartame	–	–	+	+	+

up-regulation of antioxidant enzymes (Abreu et al., 2011; Inkielewicz-Stepniak and Czarnowski, 2010; Lacorte et al., 2013; Modi et al., 2010). The antioxidant capacity of caffeine seems to be dose-dependent, as was shown in a recent study of Vignoli et al. focusing on the caffeine content in coffee beans (Vignoli et al., 2011). On the other hand, in young healthy volunteers, caffeine intake decreased the anti-inflammatory cytokine interleukin-10 and increased oxidative stress (Tauler et al., 2013).

Only few studies have examined the possible harmful effects of Coca-cola beverages on male reproductive organs. Decades ago the effects of different types of Coca-cola on sperm were analyzed *in vitro*. Umpierre et al. (1985) observed reduced sperm motility after 1 min incubation with different Coca-cola drinks. Majority of the effect was attributed to low pH, but since different formulations of Coca-cola showed different action, authors stated that the "secret formula" could also play a role as a cofactor (Umpierre et al., 1985). Two years later, a different study using transmembrane migration method did not prove the spermicidal effect, even after 1 h of incubation (Hong et al., 1987). In view of these controversial studies, both obtained from *in vitro* experimental conditions and focused mostly on sperm functions and motility, the current study was designed to elucidate possible effects of Coca-cola on the oxidative status of the testicular tissue and its endocrine function. In a previous study, Celec and Behuliak showed in male rats that *ad libitum* intake of various cola-like beverages for 3 months slightly increased both testosterone and estradiol in plasma when compared to the tap water drinking control group (Celec and Behuliak, 2010). With differences between groups just above the level of statistical significance, the study might have been too short to show convincing effects on plasma testosterone. Thus, the main aim of the current study was to attempt to reproduce the effects of intake of commercially available Coca-cola beverages with varying content of sweetener and caffeine for 6 months on testosterone production and oxidative status in testes.

2. Objects and methods

2.1. Animals

Fifty adult male Wistar rats (12 weeks old, mean body weight 358 ± 17 g) used in our experiment were purchased from AnLab (Prague, Czech Republic). Upon arrival, rats were allowed to acclimate for two weeks. Animals were kept in a controlled environment with 12/12 light/dark cycle and had *ad libitum* access to standard diet during the entire duration of the experiment (20022 KMK-20, Eypy LtD., Bruzovice, Czech Republic). This experiment was approved by the ethics committee of the Institute of Molecular Biomedicine, Comenius University in Bratislava, Slovakia.

Animals were randomly divided into five experimental groups ($n = 10$ in each group) as follows: the control group (CTRL) drinking only tap water (public water supply, Bratislava, Slovakia), the COLA group drinking only regular Coca-cola, caffeine-free (CF) group drinking only caffeine-free Coca-cola (note that caffeine-free Coca-cola does not contain glucose/fructose syrup, but artificial sweeteners, similarly to Coca-cola Zero and Light, see Table 1 for more information), the ZERO group drinking only Coca-cola Zero and the LIGHT group drinking only Coca-cola Light *ad libitum*. The main differences in Coca-cola composition are shown in Table 1. CO_2 bubbles were removed from the Coca-cola beverages before filling drinking bottles by vigorous hand shaking for 10 min and then the bottles were left 30 min opened. The amount of water/Coca-cola into drinking bottles was checked and refilled every day, fresh commercial bottles were opened as needed, but at latest within 48 h (fully unused commercial bottles were kept closed at room temperature 21–25 °C).

After 6 months animals were sacrificed, body weight and the weight of the testes were recorded. Samples of testes were snap frozen for RNA isolation and biochemical analyses, and stored at −80 °C until further analyses. Blood was collected after midline incision to abdomen through aorta using 20G intravenous flexible cannula, (BD Venflon™, Pulimedical LtD., Ivanka pri Dunaji, Slovakia) into K_3EDTA and heparin tubes. After collection the blood samples were centrifuged 5000 rpm for 5 min and obtained plasma aliquots were stored at −80 °C until further analysis.

2.2. Biochemical analyses

For determination of markers of oxidative stress and antioxidant status, samples of testes (0.2 g) were homogenized with 2 ml of ice-cold phosphate buffered saline (PBS, pH = 7.2), using TissueLyser II homogenizer (Qiagen, Hilden, Germany). The sample homogenates were then centrifuged (4000*g*, 4 °C for 10 min) and the supernatants were used for further analyses.

Protein concentrations in samples were estimated using the commercial bicinchoninic acid kit (Fermentas, Vilnius, Lithuania). We followed the instructions of the manufacturer using bovine serum albumin standard for construction of the calibration curve.

Levels of testicular advanced glycation end products (AGEs) were assessed fluorometrically ($\lambda_{ex} = 370$ nm and $\lambda_{em} = 440$ nm) after addition of PBS to samples according to Munch et al. (1997).

Fructosamine concentrations were evaluated using San-Gil et al. protocol with some modifications (San-Gil et al., 1985). Testicular homogenates and standards were transferred into microplate, incubated with nitro blue tetrazolium at 37 °C for 15 min and specific absorption was measured at 530 nm. Calibration curve was constructed with 1-deoxy-morpholino-D-fructose.

Advanced oxidation protein products (AOPPs) were measured spectrophotometrically at 340 nm, as previously described (Tothova et al., 2012; Witko-Sarsat et al., 1996). Concentration of AOPP was determined on the basis of chloramin T with potassium iodide used for the calibration curve.

Lipid peroxidation in samples was quantified by analysis of thiobarbituric acid reactive substances (TBARSs) according to established protocol (Behuliak et al., 2009). The specific fluorescence ($\lambda_{ex} = 515$ nm and $\lambda_{em} = 553$ nm) was measured using 1,1,3,3-tetramethoxypropane as standard. Our data were expressed as malondialdehyde equivalents (μmol/g protein).

Antioxidative status of the testicular tissue was measured by established spectrophotometric methods for total antioxidant capacity (TAC) and the ferric reducing ability of the tissue (FRAT) as described previously (Benzie and Strain, 1996; Erel, 2004; Tothova et al., 2012). Into 200 μl of warmed (37 °C) FRAT reagent (fresh mixture of 3 mol/l acetate buffer, 10 mmol/l tripyridyl-s-triazine dissolved in 40mmo/l HCl; 20 mmol/l FeCl3.6H2O and H2O in ratio 1:1:1:9, respectively) 20 μl of samples were added. After 4 min of incubation at room temperature the absorbance was measured at 593 nm. FeSO4.7H2O was used for the construction of calibration curve. For TAC determination, 50 μl of samples were mixed with 200 μl of acetate buffer (pH 5.8) and the initial absorbance was measured at 660 nm. After the addition of 20 μl of solubilized 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in acetate buffer with H_2O_2 and incubation for 5 min, the absorbance at 660 nm was measured again. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for the construction of the calibration curve.

Glutathione (GSH) and glutathione disulfide (GSSG) levels were analyzed as described by Arana et al., 2006 (Arana et al., 2006). The specific fluorescence for GSH and GSSG was $\lambda_{ex} = 350$ nm and $\lambda_{em} = 460$ nm. Concentration of GSH was calculated based on reduced glutathione calibration curve, the GSSG levels were determined based on oxidized glutathione calibration curve.

All measurements were done on Saphire II instrument (Tecan, Grödig Austria) and all chemicals and reagents were purchased from Sigma–Aldrich (Munich, Germany), if not stated otherwise.

2.3. Determination of plasma and testicular concentrations of testosterone

The plasma levels of testosterone were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Brno, Czech Republic). The intra and inter-assay variations were 9% and 8%, respectively.

Testosterone concentrations in testes were measured by using specific radioimmunoassay as described previously in detail (Tothova et al., 2012). The [1,2,6,7-^{3}H]-testosterone (Amersham Biosciences, Chalfont, UK, specific activity