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Influence of hesperidin and vitamin C on glycemic parameters, lipid profile, and DNA damage in rats treated with sucrose overload

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ABSTRACT

We evaluated the influence of hesperidin and vitamin C (VitC) on glycemic parameters, lipid profile, and DNA damage in male *Wistar* rats treated with sucrose overload. Rats were divided into six experimental groups: I-water control; II-sucrose control; III-hesperidin control; IV-VitC control; V-co-treatment of sucrose plus hesperidin; VI-co-treatment of sucrose plus VitC. We measured the levels of triglycerides, total cholesterol, HDL-c, LDL-c, fasting glucose, and glycated hemoglobin (A1C). DNA damage was evaluated in blood and brain cells using the comet assay and the micronucleus test was used to evaluate chromosomal damages in the rat bone marrow. Co-treatment with VitC, but not with hesperidin, normalized the serum glucose. No effect of co-treatments was observed on A1C. The co-treatment with VitC or hesperidin did not influence the lipid profile ($p>0.05$). Rats co-treated with hesperidin had a significantly lower DNA damage level in blood ($p<0.05$) and brain ($p<0.05$). Rats treated with VitC only, but not those co-treated with VitC plus sucrose, had significantly higher DNA damage in brain ($p<0.05$). No significant differences were observed in the results of micronucleus test ($p>0.05$). Hesperidin and VitC showed different effects on sucrose and DNA damage levels. While VitC lowered the serum glucose, hesperidin reduced the DNA damage.

Key words: DNA damage, hesperidin, glycated hemoglobin, lipid profile, sucrose, vitamin C.

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INTRODUCTION

Hesperidin is a flavonoid found in large amounts in citric fruits. It has various biological effects on human body, including antioxidant action and insulin-sensitizing activity (Li and Schluesener 2017). Ascorbic acid, commonly known as vitamin C (VitC), is the main hydrophilic antioxidant of human plasma (Frei et al. 1989). It is classically known that flavonoids and VitC cannot be produced by the human body and, therefore, must be consumed through the diet. Orange juice is particularly known as a source of VitC (Franke et al. 2004) and contributes greatly to the intake of flavonoids in some populations, such as the Brazilian population (Arabbi et al. 2004). Moreover, VitC and orange juice can reduce oxidative stress-associated DNA damage. On the other hand, VitC can also act as a pro-oxidant agent, while in high concentrations reacts with metals such as iron and copper (Franke et al. 2005, 2006). Similarly, depending on dose and molecular environment, hesperidin can show antioxidant (Adefegha et al. 2017, Hemanth Kumar et al. 2017) or pro-oxidant (Zhang et al. 2015a, b) activity.

A large epidemiologic study has shown that VitC concentrations were inversely associated with glycated hemoglobin (A1C) levels (Kositsawat et al. 2011). In addition, VitC has been shown to modulate glucose metabolism. The oxidized form of VitC, dehydroascorbic acid (DHAA), easily cycles back to the reduced form, and is structurally similar to glucose. DHAA entry in cells has been proposed to be mediated by the glucose transporters (Rumsey et al. 1997). Moreover, VitC impacts blood glucose levels (Al-Shamsi et al. 2007). Similarly, several classes of flavonoids modulate the transport of VitC and glucose (Song et al. 2002). However, little is known about the effect of the hesperidin on glucose and lipid metabolisms.

The different modes of action of hesperidin and VitC on human physiology indicate diverse health effects. Furthermore, there are few studies addressing the impact of VitC or hesperidin in concentrations similar to those found in citrus fruits on sucrose consumption and genomic stability. Therefore, the aim of this study was to evaluate the influence of hesperidin and VitC, in concentrations similar to those found in orange juice, on glycemic parameters, lipid profile, and DNA damage in male *Wistar* rats treated with sucrose overload.

MATERIALS AND METHODS

ANIMALS

Male *Wistar* rats (90 days old; ~250g each) were kept in individual cages in the following condition: light/dark cycle of twelve hours; 22±3ºC; 60% humidity. All animals received chow for laboratory animals (Purina Labina®, normocaloric diet) *ad libitum.* Weights of the animals and the food intake were recorded. All procedures adopted in this study followed the guidelines for the care and use of animals according to the Brazilian regulations for animal studies (Law No. 11794/1999). The experimental procedures applied in this study were approved by the Ethics in Research Committee of Universidade de Santa Cruz do Sul – UNISC (protocol 2503/10).

EXPERIMENTAL DESIGN

After 2 weeks acclimatizing, the rats were randomly divided into 6 groups (4-6 rats each): I) water control; II) sucrose control; III) hesperidin control; IV) VitC control; V) co-treatment of sucrose plus hesperidin; VI) co-treatment of sucrose plus VitC. The treatments were administered via drinking water *ad libitum*.

Based on Brito et al. (2007), we treated the animals during 60 days with 10% sucrose, without noticeable increases in glucose levels. Then, the concentration of sucrose was increased to 34%, for more 60 days, similarly to the study performed by Glendinning et al. (2010). The dose of hesperidin

(Sigma H5254) was 0.3 g/L and the dose of VitC (Sigma A4544) was 0.5 g/L. These doses were based on the average levels of these nutrients in orange juice (USDA 2005, Vanamala et al. 2006). Dilutions containing hesperidin or VitC alone or in combination with sucrose were prepared daily, and those containing only sucrose, thrice a week. Of note, both VitC and hesperidin cross the blood-brain barrier (Lam and Daniel 1986, Agus et al. 1997, Terpstra et al. 2006, El-Sayed et al. 2008, Khan and Parvez 2015). At the end of the study, the animals were euthanized by decapitation using a rodent guillotine, according to standard animal experimentation procedures.

BIOCHEMICAL PARAMETERS

The levels of 12h fasting glucose of rats were evaluated weekly using a portable glucometer (Accu-Check Advantage®). In order to validate the results with the portable glucometer, tests were performed in a reference laboratory to check blood glucose at the end of the experiment, using a commercial method, according to manufacturers' specifications (BioSystems SA, Barcelona, Spain). A1C was measured using HPLC method in the Bio-Rad Variant II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The levels of triglycerides, total cholesterol, HDL-c, and LDL-c were determined in serum according to conventional laboratory techniques using reagents from BioSystems (BioSystems SA, Barcelona, Spain).

DNA DAMAGE EVALUATION

The comet assay was used to determine the levels of primary DNA damage in blood and brain samples of the animals. Brain samples were collected at hippocampal region. We evaluated the hippocampus because previous studies indicate that the levels of DNA damage in hippocampal cells are significantly influenced by nutritional factors (Kruman et al. 2002, Haripriya et al. 2005, de Assis et al. 2009, Shirpoor et al. 2009, Molz et al. 2016). In addition, the comet assay with hippocampal cells is a well-established methodology in our group (Molz et al. 2016). The assay was performed according to Singh et al. (1988), with adaptations detailed described in previous studies published by our group (Ellwanger et al. 2015, Molz et al. 2016, Franke et al. 2017). Briefly, one hundred nucleoids ("comets") per sample were randomly selected and analyzed using a conventional microscope. The damage was determined by the classification of the comets into five classes of DNA migration: damage 0 (no tail) to damage 4 (severe damage/maximally long tail). The total score (damage index, DI) for 100 comets ranged from 0 (no damage) to 400 (severe damage) and was obtained by the sum of the results from the multiplications of the number of comets by the corresponding value to each class of damage, as follows: DI, considering 100 comets = (*n* of class 1 comets x 1) + (*n* of class 2 comets x 2) + (*n* of class 3 comets x 3) + (*n* of class 4 comets x 4).

The micronucleus test was used to evaluate chromosomal damages in the rat bone marrow. At the end of the experiment, the femurs of the animals were removed and slides were prepared as described previously by our group (Prá et al. 2008, Franke et al. 2017). Briefly, micronuclei were assessed in 2000 polychromatic erythrocytes (PCE) as a marker of chromosomal damage. The ratio of {PCE/[normochromatic erythrocytes (NCE) + PCE]} was evaluated in 1000 cells as a marker of the potential toxicity of the tested treatments.

STATISTICAL ANALYSIS

Data were analyzed and figures were plotted using Graphpad Prism v 5.1 (Graphpad Inc, San Diego). The variables were compared using the two-way analysis of variance (ANOVA), allowing the simultaneous evaluation of the effect of sucrose

and the treatment with hesperidin or VitC. In the case of significant difference regarding to sucrose or treatment (control, hesperidin or VitC), Bon-Ferroni post-hoc test was applied to compare groups. When the interaction was significant, t-test was used to compare groups. The data were checked for normality before statistical evaluation. A p value <0.05 was considered as significance level for all tests.

RESULTS

Liquid ingestion did not differ significantly between groups, and nor hesperidin or VitC altered the intake of sucrose (data not shown). Figure 1 shows the level of serum glucose and A1C in rats co-treated with sucrose plus hesperidin or VitC. Increased levels of serum glucose and A1C were observed in sucrose control. The co-treatment with VitC, but not with hesperidin, normalized the serum glucose (Figure 1a). No effect of co-treatments on A1C was observed (Figure 1b).

No noticeable changes were observed in the lipid profile of the animals, in spite of an increase in tryglicerides induced by sucrose treatment. The co-treatment with VitC or hesperidin did not influence the lipid profile (Figure 2).

Figure 3 shows the level of DNA damage in blood and brain samples by the comet assay and the results of micronucleus test in the bone marrow. Sucrose treatment induced more DNA damage in both tissues evaluated, although significantly only in brain (Figure 3b). The rats co-treated with hesperidin had a significantly lower DNA damage level in blood and brain (Figures 3a and 3b). Rats treated with VitC only, but not those co-treated with VitC plus sucrose, had significantly higher DNA damage in brain (Figure 3b). No significant differences were observed in the results of micronucleus test (Figure 3c).

DISCUSSION

VitC co-treatment showed no significative effect on A1C. However, this treatment reduced the level of fasting glucose in relation to sucrose treatment, normalizing it to the same levels of the water control. Based on the measured liquid intake and animal weights, we estimate the average intake of VitC was approximately 35 mg/kg per day. In agreement with the observed reduction in fasting glucose by VitC in our study, other studies showed that 10 to 200 mg/kg-day VitC can reduce glucose levels in streptozotocin-induced rat model of diabetes (Al-Shamsi et al. 2007, Alsaif 2009). Interestingly, there is evidence indicating that VitC can also reduce the levels of glucose in humans (Afkhami-Ardekani and Shojaoddiny-Ardekani 2007, Ashor

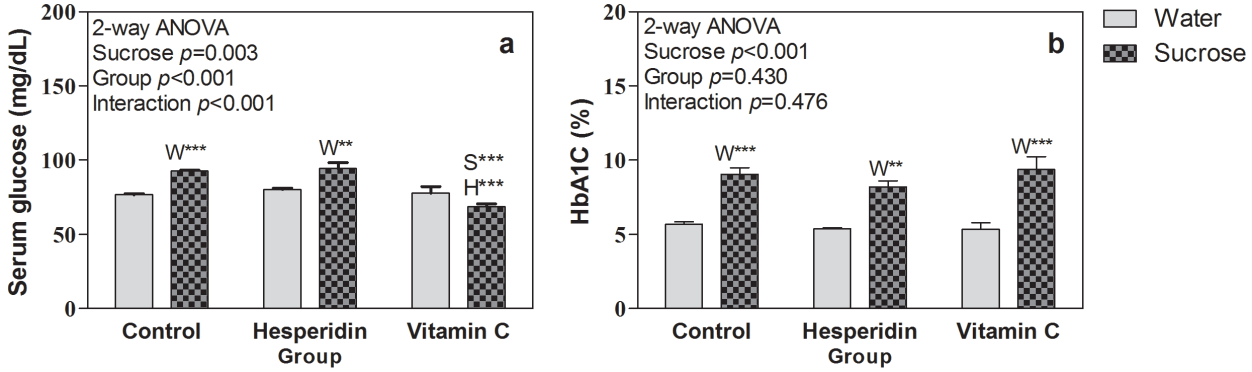

Figure 1 - Influence of vitamin C and hesperidin on fasting glucose (**a**) and glycated hemoglobin (A1C) (**b**) in rats treated with sucrose. p: level of significance according to post-hoc test in two-way ANOVA test in relation to water control (W), sucrose control (S), and hesperidin (H) at **p<0.01 and ***p<0.001.

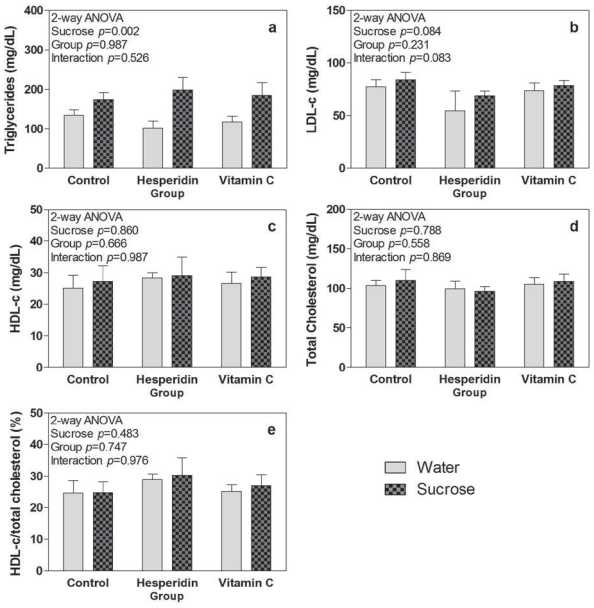

Figure 2 - Influence of vitamin C and hesperidin on triglycerides (**a**), low density lipoprotein cholesterol (**b**), high density lipoprotein cholesterol (**c**), total cholesterol (**d**), and high density lipoprotein cholesterol/total cholesterol (**e**) in rats treated with sucrose. Resuts of two-way ANOVA test.

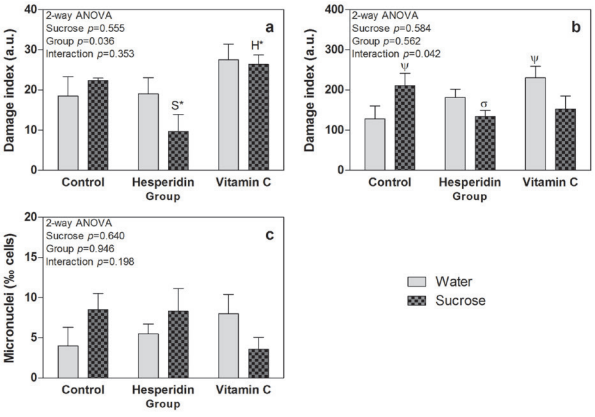

Figure 3 - Influence of vitamin C and hesperidin on damage index in blood (**a**) and brain (**b**) samples, and on micronuclei in bone marrow (**c**) of rats treated with sucrose. *p*: level of significance according to post-hoc test in two-way ANOVA test in relation to sucrose control (S) and hesperidin (H) at **p*<0.05. In the case of significant interaction, Greek letters indicate statistical significance according to t-test in relation to water (ψ) or sucrose control (σ) at *p*<0.05.

et al. 2017). The mechanisms associated with the potential capacity of VitC to reduce glucose levels are poorly understood. However, according to Afkhami-Ardekani and Shojaoddiny-Ardekani (2007), the antioxidant action of VitC could improve endothelial function and reduce oxidative stress, contributing in this way to the reduction of insulin resistance. More studies are needed to elucidate this open question.

Regarding hesperidin, we did not observe a significant reduction in glycemia or A1C. Diferently, Toumi et al. (2009) have shown glucose reduction induced by hesperidin in pregnant streptozotocin-induced diabetic mice. In agreement, a recent study performed by Iskender et al. (2017) also showed a reduction in glucose levels after hesperidin administration in a streptozotocin-induced diabetes rat model. However, these studies were performed with animal models of drug-induced diabetes, quite different from our experimental model. This difference and other particularities of the studies may potentially explain the different results obtained by us in comparison to these authors (Toumi et al. 2009, Iskender et al. 2017).

Sucrose treatment significantly increased primary DNA damage in brain samples. We have already discussed the effects of sucrose on the levels of DNA damage in Franke et al. (2017). In the present study, it was observed an increase in the level of DNA damage of brain cells caused by the administration of VitC. If we extrapolate the average intake of VitC in the control group (35 mg/kg per day) to a 70kg human being, the intake would be about 20% higher than the Tolerable Upper Level Intake (UL) dose for VitC (2 g/day to healthy adult individuals). UL is the maximum tolerable intake (of some nutrient) for a human being without adverse health effects (IOM 2000). We previously have shown a pro-oxidant effect of VitC in mice, especially when administrated in high doses (Franke et al. 2005, 2006). The present results highlight the pro-oxidant effect of VitC in rats, at least when this micronutrient is used in high doses.

Hesperidin was shown to be antioxidant protecting against free radicals-induced oxidative

damage *in vitro* (Kalpana et al. 2009), as well as acting as DNA photo-damage repair enhancer *in vivo* (Jin et al. 2011). In our study, hesperidin co-treatment reduced the DNA damage to a lower level than that observed in sucrose treatment group, in blood and brain samples. Thus, our data reinforce the antioxidant effect of hesperidin previously described by different authors (Adefegha et al. 2017, Hemanth Kumar et al. 2017, Li and Schluesener 2017).

In conclusion, under the tested conditions, hesperidin and VitC showed different effects on sucrose and DNA damage levels. While VitC lowered the serum glucose, hesperidin reduced the levels of DNA damage. On the other hand, we observed a high level of DNA damage in the brain caused by VitC, reinforcing the hypothesis that, in high doses, VitC can act as a pro-oxidant agent. However, we highlight that our study was performed in animals and the effects of hesperidin and VitC in humans can be quite different. Finally, aiming to better understand the potential effects of hesperidin and VitC on A1C, we suggest testing these nutrients in different doses used in this study, as well as using different *in vitro* and animal models.

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