Antioxidant responses in hypertensive postmenopausal women after acute beetroot juice ingestion and aerobic exercise: a randomized, double blind, and placebo-controlled trial

Abstract: This study aimed to analyze the effect of different nitrate concentrations [NO_3^-] ingestion present in beetroot juice (BJ) on salivary oxidative stress markers after acute moderate aerobic exercise performance in hypertensive postmenopausal women. For these, thirteen hypertensive postmenopausal women (58.1± 4.62 years and 27.4 ± 4.25 kg/m^2) were recruited to participate in three experimental sessions, taking different beverages: Non-Caloric orange flavored drink (OFD); Low Nitrate (Low-NO_3^-) BJ; and High Nitrate (High-NO_3^-) BJ. The participants performed moderate aerobic exercise on a treadmill, at 65–70% of heart rate reserve (HRR), for 40 min. Saliva samples were collected after an overnight fast, 10 minutes before BJ ingestion at 7:20 am (0'), 120 minutes after beverages ingestion (130'), immediately after exercise (170') and 90 min after exercise (260'). Salivary total protein (TP), catalase activity (CAT), reduced glutathione (GSH) and total antioxidant capacity by ferric-reducing antioxidant power (FRAP) concentrations were analyzed. Generalized Estimation Equation were used to compare sessions* time effects. No interaction (session*time) were found among three sessions over time. One-way ANOVA was used to compare area under the curve (AUC) of all variables over time. Catalase AUC was lower after both Low-NO_3^- and High-NO_3^- BJ consumption ($p < 0,001$); and GSH AUC was lower after High-NO_3^- BJ ($p < 0,001$) comparing with OFD. No difference in AUC after beverages ingestion were found in FRAP. In conclusion, the acute intake of BJ associated with aerobic exercise seems to decrease the production of catalase (in high-NO_3^- and low-NO_3^-) and GSH (in high-NO_3^-), besides not interfering with FRAP in hypertensive postmenopausal women medicated.

Introduction

Postmenopausal hypertensive women have increased cardiovascular risk [1], which can be caused and / or aggravated by excessive oxidative stress [2,3]. The oxidative stress is characterized by the imbalance between prooxidants and antioxidants factors in favor of prooxidants, which can lead to cell damage [4,5] due to excess of reactive oxygen species (ROS) [4,6]. ROS are involved in aging, inflammation and several cardiovascular diseases [3,4,6,7]. In postmenopausal women, hypertension is one of the most prevalent risk factors for cardiovascular disease [8], and in these patients, there was an increased antioxidant enzyme production to combat the enhance in ROS and try to maintain homeostasis [9]. The hormone estrogen, among its functions, is to increase the bioavailability of nitric oxide (NO), reduce inflammation and oxidative stress [10], and as there are changes in the trajectory of this hormone in the climacteric, NO behavior can change. Furthermore, in hypertensive patients, the NO values, as an endothelium-derived relaxing factor also have their levels altered [11,12].

An important precursor of NO is nitrate, available from two main sources: through the diet and from the NO oxidation derived from NO synthase [13]. The beetroot juice (BJ) is a common beverage that has a high nitrate concentration [14,15]. The ingestion of this beverage may generate vasodilation and reduction of blood pressure (BP) in healthy individuals [16] and hypertensive [15,17]. Other metabolic nitrate effects include interaction with mitochondrial breathing, the main metabolic pathways activation and also the oxidative stress reduction [13]. In addition, the BJ contains antioxidant compounds, like betalain pigments, that reduce linoleate damage (induced by cytochrome C oxidase) and lipid membrane oxidation (induced by hydrogen peroxide) activated by metmyoglobin and free iron [18]. Besides that, there is evidence of elimination of reactive oxygen and nitrogen species (e.g. superoxide and hydrogen peroxide) and less free radical formation by nitrite action, suggesting antioxidant effects from nitrate [14,19,20].

As for the concentration of nitrate present in the BJ necessary to perform physiological effects, it is known that plasma, salivary and urinary nitrate and nitrite increase in a manner dependent on the amount of the ingested dose [21,22], with a peak occurring in approximately 2-3 h after the ingestion. In addition, we observed in hypertensive post menopause women there is a dose-dependent salivary nitrite increase for different nitrate concentrations in BJ [23]. However, despite these responses, different levels of nitrate present in BJ also have dose-dependent effects on exercise performance [21], but not in hypotensive responses [22,23], and it is not yet known whether the concentration of nitrate present in BJ results in some effect on oxidative stress.

Another important factor that alters oxidative responses is physical exercise. Aerobic training is able to increase the values of salivary nitrite, and these values were higher than that of plasma nitrite [24]. In a study with sodium nitrate supplementation, an increase in salivary nitrite was observed both 60 minutes after ingestion and 5 days of supplementation [25]. Some studies have shown an improvement in antioxidant defense [26,27], but, acutely, the physical exercise increased pro oxidants productions [28,29], thus, in hypertensive patients, the practice of some type of exercises should be prescribed carefully, due the increased level of pro-oxidants.

Therefore, the aim of this study was to analyze the effect of BJ intake with different concentrations of nitrate [NO_3^-] on the antioxidant response. For this, we evaluated the salivary levels of nitrite and oxidative stress markers after ingestion of BJ and the performance of moderate acute aerobic exercise by hypertensive women in postmenopause.

Methods

All study stages were carried out at Laboratory of Cardiorespiratory and Metabolic Physiology of the Federal University of Uberlândia, Uberlândia, MG, Brazil, between June and September 2018. The research was registered with Clinicaltrials.gov (NCT03620227), approved by Committee of Local Ethics (70104717.0.0000.5152), and the informed consent form was signed by all participants before the beginning of the study protocol.

The sample calculation was performed using the G.Power 3.1 software, using as a main variable the variation of the catalase found after ingestion of beetroot juice and exercise session (-33.18 ± 35 nmol / min / ml) [30]. Therefore, we found that the minimum sample should be 12 volunteers, from the use of a power of 0.80; effect size of 0.474; alpha of 0.05; considering 3 groups and 4 measures.

For the inclusion criteria, participants were required to be: being a woman between 50 and 70 years old; being in post menopause (amenorrhea for at least 12 months and [FSH]> 40mIU / mL); do not perform any type of hormonal therapy; being diagnosed hypertensive; being able to exercise on a treadmill; not having a history of food allergy that could compromise the study not being sensitive to nitrate; no history of heart attack or stroke; not be diagnosed with Diabetes Mellitus; not being a user of the drug class β-blockers and not being a smoker. For the exclusion criteria, the study adopted the inability to carry out the protocol by the volunteer, being, for reasons of exercise intolerance or juice intake, or even for not fulfilling the fast

The intervention began with anamnesis and questionnaire (short version IPAQ) application answered by the volunteers, later anthropometric measures were taken, which included: body mass (electronic scale Filizola, São Paulo, Brazil); height (Sanny fixed stadiometer, São Bernardo do Campo, Brazil); abdominal circumference (Filizola inelastic tape, São Paulo, Brazil); and body composition (biobody impedance Inbody 230, Seoul, South Korea), evaluated according to the previous description [31]. For body composition, the volunteers were instructed not to consume alcohol and drinks containing caffeine 72 hours before the exam, and not to perform vigorous physical activities 24 hours before.

Results

Two out of the 15 volunteers were excluded from the study for inability to adapt to the study protocol due to an abrupt decrease in blood pressure followed by syncope as of 15 minutes after the beginning of the first exercise session. One of the volunteers had consumed High-NO_3^- and the other Low-NO_3^-.

Then, this study was completed by 13 medicated hypertensive postmenopausal women, with mean age of 58,1 ± 4,6 years and body mass index of 27,4 ± 4,2 kg/m². The IPAQ results showed that 15% was very active, 62% active e 13% irregularly active, and there were no sedentary women. It is worth mentioning that blood pressure and nitrite concentration data from the same sample and intervention have already been published, just like the others general characteristics [23]. In this previous study, we emphasize that, as expected, salivary nitrite over time were different among the three beverages (biggest increase in High-NO_3^- BJ, very smooth increase in Low-NO_3^- BJ and no changes in OFD). In addition, we found an important post-exercise hypotension, but the BJ intake was not able to potentiate these responses.

Among the antihypertensive drugs used, 46% (6) used Angiotensin Receptor Blockers plus Diuretic polytherapy, 8% (1) Angiotensin Converting Enzyme Inhibitor plus Diuretic, and 31% (4) monotherapy with Angiotensin Receptor Blockers, 8% (1) with Diuretic and 8% (1) with Angiotensin Converting Enzyme Inhibitor. Among the other medications used, 31% (4) used Levothyroxine and 23% (3) Statins.

Table 1 shows salivary oxidative stress markers and total protein as well as the p values of interaction, intervention and time. No interaction (session* time) were found in the salivary markers. It was possible to verify the session effects on catalase (OFD didn't change; Low- NO_3^- and High-NO_3^- increased) and GSH (High-NO_3^- increased). Regarding the effect of time, we found a significant difference in all variables being that: in total protein, 170 ’time was bigger than all other times; in catalase, 170’ it was lower than baseline; in GSH the times 130’ e 170’ were greater than baseline; an in FRAP 130’ and 260’ were lower than baseline, 170’ was bigger than 130’, 260’ was lower than 170’.

Figure 2 shows the salivary oxidative stress markers over time and their respective AUCs. The AUCs of salivary catalase activity from both High-NO_3^- and Low-NO_3^- BJs session (figure 2B) were lower ($p<0.01$) than OFD session. Only salivary GSH AUC from High-NO_3^- BJ session was

lower ($p<0.01$) than OFD session (Figure 2D). No difference was found in FRAP AUCs among sessions.

Discussion

Our study investigated if acute BJ ingestion with different [NO_3^-] doses could change the salivary oxidative stress markers after a moderate aerobic exercise in hypertensive postmenopausal women. The main results were that the antioxidant responses are better in high amounts of [NO_3^-], although we also found a positive effect in catalase levels in both BJ with high and low [NO_3^-]. Catalase reduced with the intake of high- NO_3^-- and low- NO_3^--, and post moderate exercise a fall occurs. In the GSH data, we observed that its levels are maintained when there is consumption of high- NO_3^--, while non-caloric orange flavored drink without nitrate (OFD) increases. Furthermore, exercise does not alter GSH, since the moments before and after exercise session, were greater than the baseline. After drinking and 90 minutes after exercise, FRAP reduces when compared to baseline, but intervention with high- NO_3^-- or low- NO_3^-- does not differ in their responses in relation to OFD.

The acute BJ ingestion with high doses of nitrate has been studied as a potential antioxidant. These effects occur because nitrate is an important precursor of NO [21], and beetroot contains antioxidant nutrients, like polyphenoid compounds, and free radical scavengers [14,18,39]. In vitro study [40], it was observed that a shot of BJ manages to supply a large number of bioaccessible antioxidants in gastric and duodenal cells, hypothesizing that this would be an interesting strategy to increase the body's antioxidant status. Some authors showed in a study with diet control over four weeks of BJ supplementation [41] an increase in lipid peroxidation, indicating a change in oxidative behavior. Thus, our initial hypothesis was that antioxidant factors would increase acutely with high-NO_3^-, would have a lower response with low-NO_3^-, and would not change with OFD.

In this sense, in a previous study [23] with similar beverages and population, we found an increase in salivary nitrite with ingestion of high- NO_3^--, but there was no potentiation of post-exercise hypotension with the ingestion of any beverage. In addition, in a systematic review [42], it

149 is commented that BJ is rich in antioxidants that suppress the accumulation of leukocytes after
150 exercise, reducing muscle damage and that the betalain present in beetroot is able to attenuate
151 muscle damage by effects analgesic and anti-inflammatory effects.

152 Our results showed that the catalase activity decreased after exercise in both high-NO_3^- and
153 low-NO_3^- sessions and this shows that possibly nitrate had no effect (or had a partial effect) on this
154 antioxidant enzyme, but what may have reduced this enzyme are other antioxidant substances
155 present in beetroot juice. This enzyme is an antioxidant enzyme that acts as the first line of defense
156 against oxidative stress [43]. One of its functions is to catalyze the degradation or reduction of
157 hydrogen peroxide [43]. Hydrogen peroxide in high concentrations is harmful to cells and is related
158 to the pathophysiology of arterial hypertension [44]. In a preclinical study, there is evidence that the
159 production of catalase increases with the presence of ROS; however, with the presence of beet leaf
160 extract its levels are restored to baseline [45]. It was noticed that catalase responses post intense
161 exercise in amateur athletes there is an increase in its bioavailability for up to 24 hours post-
162 exercise. In heart failure patients [46], post 30 minutes of moderate continuous exercise, there is a
163 catalase increase, but after low-intensity exercise with greater volume (45 minutes), there is a
164 reduction in this enzyme. Which may indicate that the intensity of the exercise is more related to the
165 increase in oxidative stress than the volume. Regarding the ingestion of beetroot juice, in athletes
166 [30] the catalase activity increased after interval exercise, with no difference with the placebo,
167 distinguishing from the present study, in which the catalase activity reduced similarly between high-
168 NO_3^- and low-NO_3^- .

169 Another important endogenous antioxidant is GSH, which is involved in cellular protection
170 against excessive oxidative stress both directly and as an enzymatic cofactor of glutathione
171 peroxidases [47]. This is an antioxidant molecule oxidized by glutathione peroxidase (GPx), an
172 intracellular enzyme capable of reducing hydrogen peroxide with an important role in inhibiting the
173 lipid peroxidation process [43]. Our results showed that salivary GSH concentration decrease only
174 after high-NO_3^-. In addition to betanine, a beetroot is also rich in polyphenoid compounds, such as

ascorbic acid, carotenoids, phenolic acids, flavonoids[52] and bioactive betalain pigments [53], all of which are potential antioxidants [40]. This may indicate that the antioxidants present in beetroots increase the values of GSH, although the difference with placebo was observed only with a high-NO_3^-, which shows that nitrate also plays an important role. Unlike the present study, in a study with chronic intake by 7 days of 2 shots with 6.5-mmol NO_3^- beetroot juice per day with active and healthy young men [54], there was no found changes in GSH values, which may indicate that chronically beverage intake, with double nitrate concentration in two doses per day, does not modify these responses in a healthy population. When verified these responses in intense exercise in amateur athletes [55], GSH did not undergo any changes within 72 hours after exercise. However, when we observe these responses post moderate resistance exercise with active young men [56], there is an increase in plasma GSH, but without differences in salivary analysis, which may indicate greater sensitivity of blood analysis. From our results, we realized that a high-NO_3^- dose of BJ reduces GSH, probably due to the presence of a high dose of nitrate related to the presence of antioxidants and other components, such as GSH high levels and other antioxidant molecules, not evaluated here.

The last variable analyzed was the FRAP, which aims to analyze the antioxidant power [57]. In vitro study [40], using the same beetroot juice from the present study, an increase in FRAP was found after the gastric phase, with a marked reduction after the duodenal phase, which may be related to the biotransformation of antioxidants caused by the interaction with enzymes. In a review study [14], it is argued that FRAP increases after ingestion of beetroot juice, contrary to our findings since in the present study there were no differences between drinks. It is worth noting that, although we found $p < 0.001$ in the area under the curve, there was no significant value in the post hoc analysis. We have found no clinical study that analyzed FRAP associated with the intake of BJ, but in hypertensive patients, there is evidence of less production when compared with normotensive individuals [58,59]. In addition, negative correlations were found with systolic and diastolic blood pressures [59], suggesting a role for plasma antioxidant status in modulating blood pressure. As for

the present study, we found a reduction in the FRAP levels after drinking the beverages and 90 minutes post physical exercise. In trained healthy men [37], FRAP values increased after continuous exercise session and decreased 3 hours post exercise. These results corroborate our findings, considering that with the intake of beverages there was a reduction in FRAP, followed by an increase caused by the influence of physical exercise, returning to the values found just before the beginning of exercise 90 minutes after the same. In addition, in another study with physically active men [25], associating sodium nitrate supplementation with 30 minutes of moderate aerobic exercise, no FRAP modification was found. This suggests that supplementation of dissociated nitrate from foods with other compounds (e.g. beetroot juice) may interfere with the response of this variable.

In addition to the characteristics of the intervention, we must consider that the sample of this study was composed of women diagnosed with hypertension and controlled by antihypertensive drugs. We observed that 92% (12) of the volunteers used drugs that act on the renin-angiotensin system. In this sense, AT1 receptor antagonists or ACE inhibitors may have antioxidant actions by improving endothelial function [60] and reducing ROS production by reducing NADPH oxidase and regulating Cu / ZnSOD [61]. It is worth mentioning that since physical exercise was prescribed based on heart rate, we did not include users of β-blocking drugs. Another important point of the present study is the use of salivary evaluation as a way of analyzing the antioxidant profile. It is already possible to find in the literature [37] that the antioxidant profile of saliva samples was similar to that of plasma, suggesting that as an alternative and non-invasive tool, saliva may be an interesting method for studying antioxidants in sports medicine.

Among the limitations of our study, we have the use of antihypertensive drugs by all volunteers, since these drugs can exercise specific actions not analyzed. To minimize these effects, we indicated that the volunteers kept the time of day that they used the medication. In addition, with this study, we assessed only the acute effects of BJ intake, and we did not assess its isolated effects (without exercise). We also did not carry out the dietary control of these volunteers, but as a way of

minimizing this factor, they should follow a list of nitrates foods rich that should not be consumed the day before the test. In addition, we have not evaluated several other antioxidant enzymes, which could give a better response to the oxidant profile. These results can be applied specifically to hypertensive women after menopause, and cannot be stratified for other populations.

As a clinical application, we realized that the use of BJ as a treatment for hypertension has been studied in recent years [62]. There are discussions about the increase in hypertension incidence, especially low- and middle-income countries [63], and the treatment of this disease in these countries can be hampered, both by the health system and by the level of education and income population. We believe that this study can contribute to findings foods benefits that are already present in population diet and can be used to add knowledge to alternative treatments of hypertension control.

Thus, we conclude that the acute intake of beetroot juice associated with moderate-intensity aerobic exercise seems to decrease the production of catalase (in high-NO_3^- and low-NO_3^-) and GSH (in high-NO_3^-), besides not interfering with FRAP in hypertensive postmenopausal women medicated. Thus, BJ may have positive effect in oxidative stress markers independent of [NO_3^-], although we can see that high-NO_3^- can have better results in the antioxidant response, since it produced positive responses in two variables.