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HEALTH SCIENCES

Whole purple grape juice increases nitric oxide production after training session in high level beach handball athletes

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Abstract: Aims to evaluate the effect of whole purple grape juice intake in the recovery of oxidative stress, inflammation and muscle injury after an intense training session. Fifteen high level men athletes were randomly distributed in supplemented (GJG; n=8; 28.7 ± 3.5 years) or control group (CG; n=7; 24.8 ± 2.7 years). 400 ml of juice or water was ingested immediately before (200 ml) and after (200 ml) a training session. Blood samples was collect before and post-training session as well as 180 minutes after this session (recovery) to analysis of creatine kinase (CK), lactate dehydrogenase (LDH), C-reactive ultrasensitive protein (CRP), malondialdehyde (MDA) and nitrite. The nitrite values at the end of recovery moment of the GJG group were significantly higher than the same moment in the CG group ($p<0.05$), and the intra-group analysis showed a significant increase in nitrite values only in the GJG group in the end of recovery period moment when compared to the moments pre-training ($p<0.001$) and post-training ($p<0.05$). MDA, CRP, LDH and CK did not differ neither groups. Acute supplementation with 400 ml of grape juice increases the serum concentration of nitrite, a nitric oxide metabolite that has antioxidant activity.

Key words: ergogenic foods, exercise, inflammation, muscle injury, oxidative stress, purple grapes.

INTRODUCTION

Regular physical exercise at moderate intensity increase substantially the endogenous antioxidant capacity (Gomez-Cabrera et al. 2008) and reduces systemic inflammation as an adaptive response (Fedewa et al. 2017). On the other hand, repetitive intense and/or prolonged training leading to excessive muscle damage, systemic inflammation and oxidative stress in athletes has been documented (Rowlands et al. 2012). Although still a controversial subject, the most accepted theory of overtraining shows a systemic inflammation, accompanied by oxidative stress among the factors involved in the etiology of this phenomenon. (Carfagno & Hendrix 2014).

The beach handball is a sport characterized by many high intensity actions with large number of repetitions, the game is played on sand beach and two sets of 10 minutes each, which makes very intense game. This difficulty is critically enhanced by the fact that at competitions is common for a team play more than one match on the same day. There are no stops for time-outs, and when a goal is scored against a team, the goalkeeper typically places the ball back in play immediately. The game is designed so that counter-attacks are exploited at all times. During a game, athletes reach high heart rate and blood lactate production during the game (near the anaerobic threshold) (Silva et al. 2016).

In this sense, antioxidant and anti-inflammatory foods and nutritional supplements has been included in dietary of athletes aiming to minimize inflammation and oxidative stress induced by strenuous exercise, so there are reports indicating that cherry juice (Howatson et al. 2010), honey (Tartibian & Maleki 2012), blueberry shake (McLeay et al. 2012), tomato (Tsitsimpikou et al. 2013), beet (Domínguez et al. 2018), Cocoa (Decroix et al. 2018) promoted either reduced muscle damage, oxidative stress or systemic inflammation, all of which resulted in improved physical performance. Although promising, these studies were made with cyclic sports (runners, swimmers, cyclists), so there are few data with team sports. In these sports, the number of accelerations, decelerations and changes of direction occur minimally compared to acyclic sports such as beach handball. These actions lead to much greater muscle wasting, which increases the physiological demand of these modalities (Silva et al. 2016), so studies of foods with ergogenic effects are pertinent.

Purple grapes and derivatives are a class of foods with particular antioxidant and anti-inflammatory potential due to rich composition in polyphenolic compounds, such as proanthocyanidins, catechins and resveratrol (Dani et al. 2007) and has the ability to increase plasma levels of superoxide dismutase, catalase (Ribeiro et al. 2018) and total antioxidant capacity (Toscano et al. 2015). In addition, studies have shown that grapes also act in the antioxidant system, increasing nitric oxide production, which increases the bioavailability of this gas and facilitates antioxidant action through nitrite peroxide [to transform the superoxide anion (O2-) into nitrite peroxide (NOO-)] (Nematbakhsh et al. 2013, Zolfaghari et al. 2015, Kaushik 2016). Toscano et al. (2015) confirmed these anti-inflammatory and antioxidant effects with daily supplementation with purple grape juice at 10 ml/kg for 28 days leading to improved performance, however the study was once again with cyclic sports (runners).

Because of these features, speed recovery after physical exertion represents an ergogenic possibility of high impact for this sport. For this reason, this study was conducted to evaluate the effect of purple grape juice intake in the recovery of oxidative stress, inflammation and muscle injury few hours after an intense and long-term training session in high level athletes of the male brazilian beach handball team.

MATERIALS AND METHODS

Subjects

This was randomized and controlled trial. All fifteen male athletes of the Brazilian national beach handball team participated of this experimental randomized controlled trial. They were randomly distributed in supplemented group with integral purple grape juice (GJG; n=8; 28.7 ± 3.5 years) or a control group that ingested water (CG; n=7; 24.8 ± 2.7 years). Could not participate or would be excluded from the study athletes who had some infectious process at the time of the study, who had complained of injury or symptoms of muscle injury before or during study and those who did not participate in the training assiduously. However, these criteria did not need to be applied to any athlete. This work was previously approved by the Ethics Committee of the Lauro Wanderley University Hospital, Federal University of Paraíba, under number 637.299/14. Participants were informed about the procedures involved and required to sign the consent form and defined according to the resolution 466/12 of the National Health Council.

Study design

The experimental design of the study is shown in Figure 1. The study was done in a training phase with loads that are particularly higher than the other times of the season. The athletes were assembled for a training phase (camping) with multiple daily sessions (two or three sessions). The experimental procedure was performed on the third day of this training phase, which corresponded to the competitive period previously to the World Championship games. The experimental protocol consisted of the athletes ingesting a single dose of grape juice or water immediately before and after a training session. Blood samples was collected to analysis of creatine kinase (CK), lactate dehydrogenase (LDH), C-reactive ultrasensitive protein (CRP), malondialdehyde (MDA) and nitrite before and post-training session as well as 180 minutes after this session (recovery).

Training

The study protocol did not modify the training program prepared by the technical committee. The team was in training camp phase, characterized by the accumulation of training loads in a short time recovery (three daily training sessions lasting 120 minutes each session and 180 minutes intervals between sessions. Experimental procedures occurred at third day of training camp, in the first session of this day. The training was done in half the morning (8:00 am), and the athletes had trained until 06:00 pm the previous day. The team was in concentration regime, so that everyone fed and went into the rooms to sleep around 9:00 pm in previous day. The training lasted 120 minutes and was characterized as technical and tactical, started with stretching / warm up and finished with a collective game. In the next three hours post-training, the athletes were rested, and in the end, they had the last collections done.

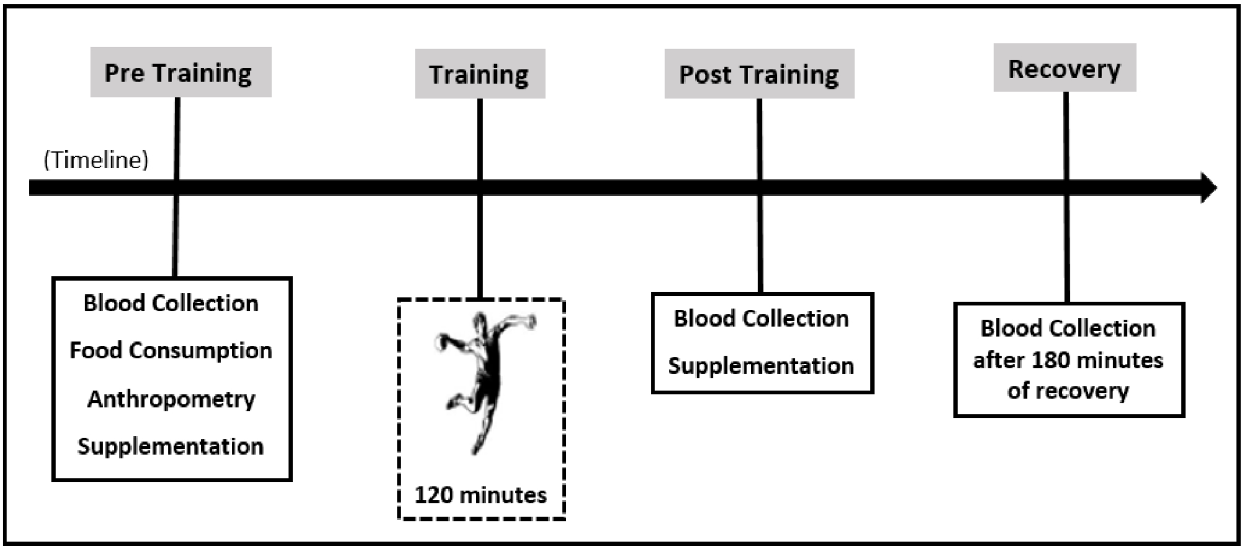

Figure 1. Study Design.

Supplementation protocol and nutritional control during the experimental procedure

The experimental group was supplemented with 400 ml of grape juice (Vinson et al. 2000) and the control group received 400 ml of mineral water. Supplementation was divided in equal volumes to be ingested before (200 ml) and in the final of training (200 ml). Throughout the experimental protocol water was provided *ad libitum* to alleviate the discomfort of the subject during the experiment. No food supplement was consumed during the experimental protocol.

Grape juice chosen to supplement the athletes was integral purple type (Aurora, Bento Gonçalves, Rio Grande do Sul, Brazil), ruby coloration, fruity aroma, soft feature and made from grapes of American origin (Bordô and Isabel). In the 200 ml portion contains 123 Kcal, 30 grams of carbohydrates, it does not contain significant amounts of protein, total fats, saturated, trans, dietary fiber and sodium. The athlete´s breakfast was typical of hotel and was held at 7am, being food sources of carbohydrates, proteins and did not have foods rich in antioxidants.

Food consumption

The dietary intake was evaluated through a 24-hour food recall (Gibson 2005) applied in two stages, before and during the experimental protocols, two of which were for weekdays, and one for the weekend, for each stage. The data were evaluated in the software Avanutri Revolution version 4.0 (Avanutri Informática Ltda, Rio de Janeiro, Brazil) and for reference of dietary intake adequacy were considered the limits proposed by the International Society of Sports Nutrition (Kreider et al. 2010).

Biochemical analyzes

Was collected 10 ml of blood for analysis of creatine kinase (CK), lactate dehydrogenase (LDH) and C-reactive ultrasensitive protein (CRP) to assess muscle damage and inflammation using specific commercial kits (Labtest - Brazil) in analyzer automatic (Labtest, LabMax 240 premium - Brazil). The malondialdehyde concentration (MDA) was determined and quantified by the reaction of thiobarbituric acid (TBARS) with the products of decomposition of hydroperoxides, according to the method described by Ohkawa et al. (1979). The concentration of nitrite was determined by the Griess reaction according to the methodology proposed by Bryan & Grisham (2007). In addition to the pre-training, post-training and 180 minutes post-training analyzes, this analyzes were also performed before the first training phase (three days before the experimental session), for purposes of characterization and verification of the physiological state of the groups.

The interpretation of the results of blood markers levels were according Cooper et al. (2017) (<300 U/L) for CK and LDH according Srividhya et al. (2017) (138.4 to 746.0 IU/L). Oxidative stress (MDA) according Kadiiska et al. (2005) (1 to 3 μM) and nitrite value there is not an established reference standard for athletes or clinical. Systemic inflammation (CRP) there are no data for athletes, so clinical standards have been adopted (<3.0 mg/L) according specific commercial kit (Labtest - Brazil).

Statistical Analyses

The results were presented as mean and standard error of the mean. Shapiro-Wilk and Levene tests were applied to verify the normality and homogeneity. The independent t test was used to compare baseline and food consumption between groups (pre-experimental values). Repeated measures ANOVA with Bonferroni post hoc was adopted to verify the responses at time × group interactions and partial eta-squared.

Data were analyzed using the IBM SPSS Statistcs 24.0, adopting significance of $p < 0.05$.

RESULTS

Pre-training characteristics of the athletes are shown in Table I. The groups were similar in age, body mass, body fat, muscle mass and BMI. The physiological status before the first day of training was similar in both groups for all blood markers analyzed. Considering that experimental procedure occurred on the 3rd day of the training phase and all athletes from both groups performed all the training until the day of the experimental session (three sessions/day), all athletes were in the same conditions of external training load. As consequence the two groups presented signs of muscle damage (high levels only for CK), without differences between them, although with LDH levels within normal range. On the other hand, the external load had not had repercussions at systemic level, since the athletes of the two groups had values within the limits of normality for the indicators of systemic inflammation and oxidative stress.

The food consumption of athletes (nutritional intake of the day before the experiment) is presented in Table II. The groups showed no differences in daily energy intake and macronutrient, however was observed higher consumption of selenium by grape juice group. Both groups showed insufficient intake of vitamin A, copper, magnesium and excess zinc.

Table I. **Pre-training characteristics of the athletes.**

	Grape juice (n=8)	**Control (n = 7)**	**p**
Age (years)	26.3±1.4	24.9±1.1	0.45
Height (cm)	1.80±0.03	1.90±0.01	0.01*
Body mass (kg)	91.1±6.7	87.4±3.8	0.65
BMI (kg/m²)	26.3±1.2	24.2±0.9	0.19
Body fat (%)	13.9±2.5	12.7±1.7	0.70
Muscle mass (%)	41.7±1.1	41.9±0.6	0.88
Daily training (minutes)	~ 270	~ 270	0.99
	Pre-Training	Pre-Training	
CK (U/L)	796.8±106.1	1000.1±243.1	0.45
LDH (U/L)	595.0±32.5	632.9±62.5	0.44
MDA (µmol/L)	1.1±0.2	1.1±0.2	0.99
CRP (mg/L)	1.5±0.3	0.7±0.2	0.26
Nitrite (µmol/L) (n=7)	8.4±0.7	9.7±1.1	0.44

Data are expressed as mean ± standard error. BMI = body mass index; CK = Creatine Kinase; LDH = lactate dehydrogenase; MDA = Malondialdehyde; CRP = C-reactive ultrasensitive protein; Independent t-test, p <0.05. The interpretation of the results of blood markers levels were according Cooper et al. (2017) (<300 U/L) for CK and LDH according Srividhya et al. (2017) (138.4 to 746.0 U/L). Oxidative stress (MDA) according Kadiiska et al. (2005) (1 to 3 µM) and nitrite value there is not an established reference standard for athletes or clinical. Systemic inflammation (CRP) there are no data for athletes, so clinical standards have been adopted (<3.0mg/L) according specific commercial kit (Labtest - Brazil).

Other micronutrients were normal (Institute of Medicine 2006).

Blood markers at pre-training, post-training and at the end of the recovery period (recovery) of the experimental session are shown in Table III. The training did not promoted change in muscle damage, since the values of CK and LDH did not change in the time or time x group interaction. Likewise, there were no systemic repercussions since CRP did not indicate an increase in systemic inflammation and MDA did not indicate an increase in lipid peroxidation in the time interaction and there were also no differences between groups.

On the other hand, the grape juice promoted some alteration in the synthesis of nitric oxide, since the grape juice group demonstrated a relevant increase of almost four times in the production of nitrite in the serum concentration of nitrite after the period of 3 hours of recovery, which was significantly higher than the values pre training and post training (time interaction), as well as in relation to the same moments of the control group (time x group interaction). The individual response of each subject (plasmatic nitrite) is shown in Figure 2.

DISCUSSION

The intake of grape juice did not promote changes in CK, LDH, MDA and CRP. On the other hand, was able to promote expressive post exercise increase in plasmatic nitrite concentration. The training session did not promote additional wear in relation to the pre-training moments, in relation to muscle damage, systemic inflammation and

Table II. Food consumption of athletes.

	Grape juice (n=8)	Control (n=7)	p
Total energy intake (Kcal)	5376.7±717.3	6088.7±590.6	0.45
Carbohydrates (g/day)	710.9±143.3	702.4±94.6	0.96
Carbohydrates (%)	49.8±2.7	46.6±4.2	0.54
Proteins (g/day)	287.9±31.8	337.8±53.2	0.45
Proteins (%)	23.6±2.5	22.5±2.3	0.74
Lipids (g/day)	147.1±18.2	208.5±34.3	0.15
Lipids (%)	26.4±1.5	30.7±2.8	0.22
Vitamin A (RE)	864.9±126.8	713.5±117.4	0.40
Vitamin C (mg)	367.6±65.7	427.0±52.4	0.49
Vitamin E (mg)	15.0±2.5	26.7±5.8	0.10
Copper (mcg)	1.1±0.0	2.6±1.0	0.19
Zinc (mg)	13.1±2.1	19.5±4.3	0.23
Selenium (mcg)	147.7±18.6	1.6±0.3	0.0002*
Magnesium (mg)	298.8±27.7	334.0±47.0	0.59

Data are presented as mean ± standard error. *difference between groups, p = 0.0002 independent t test.

oxidative stress. On the other hand, grape juice promoted increased nitric oxide production, indicated by increased endothelial activity and this increased bioavailability of nitric oxide acts as an antioxidant (Pacher et al. 2007).

The increased blood flow promoted by exercise results in a phenomenon called *shear stress* on the blood vessel wall, which activates receptors that in turn activate the endothelial enzyme NO synthase (eNOS) and catalyzes an increase in nitric oxide production, which one of the metabolites is nitrite (Green et al. 2004). Despite this theoretical possibility, similar to the present study, exercise-associated L-arginine intake also showed a significant increase in nitric oxide production (Lima et al. 2012). Regarding the isolated effect of grape juice in stimulating the production of nitric oxide (without physical exercise), other studies had already shown an increase in the production of this gas after the consumption of grape juice and beetroot, but not in athletes. Among them, Takahara et al. (2005) demonstrated that the ingestion of 3 ml/kg of a new non-alcoholic beverage made of red wine vinegar and grape juice can activate the nitric oxide synthase activity in rats. Freedman et al. (2001) demonstrated that consumption of purple grape juice (7 ml/kg/day) for 14 days increased platelet-derived NO production from 3.5±1.2 to 6.0±1.5 pmol/10, result similar to that found in the present study.

As a source of nitrate and corroborating our data, Clifford et al. (2017) showed that after consumption of 250 ml of beetroot juice (BTJ), 300 g of whole beetroot (BF) or a placebo drink, the plasma nitric oxide reached peak concentrations 2 h post-ingestion in both experimental groups (BTJ - 163.7±46.9 and BF 189.4±72.8 µmol/L; p < 0,001), both groups started with nitric oxide concentration next to 55 µmol/L. Confirming this data, Clifford et al. (2015) showed in a literature review that beetroot ingestion provides a natural means of increasing *in vivo* nitric oxide (NO) availability and has emerged as a potential strategy to prevent and manage pathologies associated with diminished NO bioavailability, notably hypertension and endothelial function. Beetroot is also being considered as a promising therapeutic treatment in a range of clinical pathologies associated with oxidative stress and inflammation. Its constituents, most notably the betalain pigments, display potent antioxidant, anti-inflammatory and chemo-preventive activity *in vitro* and *in vivo*.

Increased bioavailability of nitric oxide may have beneficial repercussions for athletes. High concentrations of nitric oxide could provide ergogenic effects, improving tolerance to resistance exercise in endurance exercise, as shown in literature review (Jones 2014), improve muscle power output via a mechanism involving a faster muscle shortening velocity in high-intensity exercise (Domínguez et al. 2018), by increasing the travelled distance (3.4 ± 1.3%) in a football match after performed high-intensity endurance exercise using the Yo-Yo IR1 test after having increased nitrite levels (632±66 nM in experimental group vs. 186±13 nM in placebo group; p < 0.001) (Nyakayiru et al. 2017) where thirty-two male trained soccer players improved performance. Totzeck et al. (2012) also showed that eleven athletes improved performance by increasing serum levels of nitrite after performed intense stationary bicycle ergometer exercise, baseline plasma nitrite levels correlated with lactate anaerobic thresholds (r = 0.65; p = 0.001, n = 22) and with endothelial function as assessed by flow-mediated vasodilation (r = 0.71; p = 0.0002). Dreissigacker et al. (2010) investigated a potential link between plasma nitrite synthesis and bioavailability and oxidative stress in the circulation of subjects performing highly intensive endurance exercise. Twenty-two male healthy subjects cycled at 80% of their

Table III. Behavior of blood markers at pre-training, post-training and at the end of the recovery period (recovery).

	Grape juice (n=8)	Control (n=7)
CK (U/L)		
Pre-training	796.8±106.1	1000.1±259.8
Post-training	851.3±107.8	966.1±202.4
Recovery	807.8±102.6	997.1±260.9
d – Cohen	0.50 / 0.10	0.15 / 0.01
LDH (U/L)		
Pre-training	595.0±32.5	632.9±66.9
Post-training	610.8±19.4	613.6±54.6
Recovery	640.9±29.5	622.3±77.9
d – Cohen	0.53 / 1.47	0.32 / 0.14
CRP-us (mg/L)		
Pre-training	1.5±0.3	0.7±0.2
Post-training	1.5±0.3	0.7±0.2
Recovery	1.5±0.4	0.6±0.2
d – Cohen	0.00 / 0.00	0.00 / 0.50
MDA (µmol/L)		
Pre-training	1.1±0.2	1.1±0.2
Post-training	1.0±0.2	0.9±0.6
Recovery	0.9±0.8	1.4±0.3
d – Cohen	0.50 / 0.27	0.37 / 1.13
Nitrite (µmol/L) (n=7)		
Pre-training	8.4±0.7	9.7±1.2
Post-training	16.5±2.8	15.4±1.4
Recovery	32.3±7.2$^{*\#}$&	17.2±2.4
d – Cohen	3.09 / 3.28	4.46 / 3.60

Data are presented as mean ± standard error. MDA = malondialdehyde; CRP-us = C-reactive ultrasensitive; CK = Creatine Kinase; LDH = lactate dehydrogenase; d – Cohen = Effect size (Post-training vs Pre-training / Recovery vs Pre-training). ANOVA one way with Bonferroni posttest for comparison between groups and ANOVA for repeated measures within groups. *Recovery vs Pre-training (p<0.001). #Recovery vs Post-training (p<0.05). &Recovery Grapre Juice vs Recovery control (p<0.05).

maximal workload, and the findings provide evidence of a favorable effect of nitrite on high-intensive endurance exercise, because nitrite plasma concentration decreased significantly during the trial (767 ± 234 nM at rest for 674 ± 204 nM end of exercise) leading to a drop in physical performance.

Another possible benefit of nitric oxide is its antioxidant function. Nitric oxide has been proposed as a potent antioxidant, since it has the capacity to transform the superoxide anion (O2$^-$) into peroxynitrite (NOO$^-$), which is less reactive (Pacher et al. 2007). This antioxidant capacity was demonstrated by Pacher et al. (2007) in a literature review, showing that the increase in the synthesis and bioavailability of nitric oxide combat oxidative stress and decreased membrane fluidity caused by extenuating acute exercise.

Finally, a third possible benefit of nitric oxide is its vasodilatory action. Purple grape juice also has been proven to increase the production of nitric oxide, being this gas, a potent vasodilator produced by human tissues, such as the endothelium (Folts 2002). This effect is attributed to their rich polyphenol composition, with the main component being flavonoids (Dohadwala & Vita 2009); however, there is no evidence that this vasodilation may have an ergogenic effect on athletes.

It is interesting to note that the training was not able to generate muscle damage, systemic inflammation and oxidative stress in the athletes, since the levels of CK, LDH, CRP and MDA did not change in any of the groups. It should be noted that the athletes were already on the third day of the training phase, and that on the previous two days there were expressive loads of training with three sessions/day.

As they were with CK levels indicating muscle damage at pre experimental moment, it is possible that the experimental session did not provide additional damage. On the other hand, muscle wasting was only at the local level, since the athletes did not present systemic disturbances, inflammation and oxidative stress, which is considered an indicator that excessive training loads are generating deleterious repercussions at systemic levels (Coppalle et al. 2019) so the experimental session did not modify these variables.

Our choice to perform the experimental protocol on the third day of the training phase was precisely to find the athletes with some accumulated fatigue, which could be a better experimental model to test the antioxidant

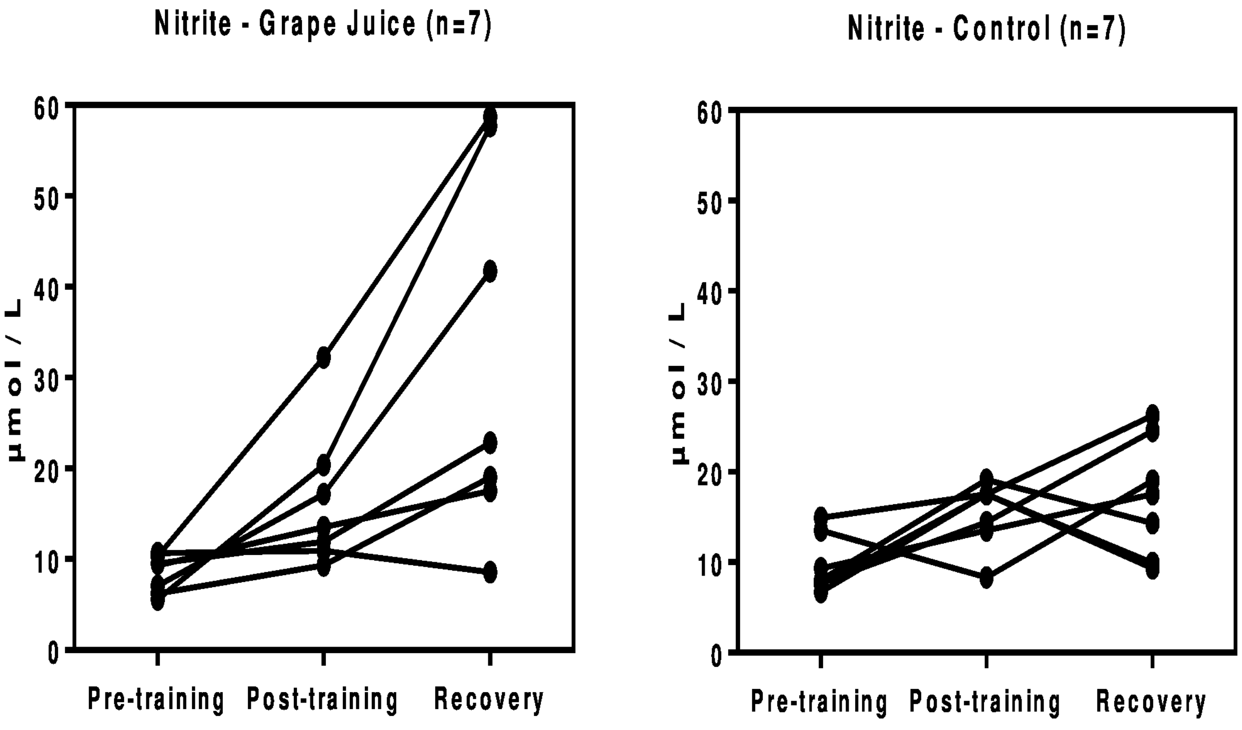

Figure 2. The individual response of each subject (plasmatic nitrite).

activity of grape juice. However, we find different results than expected for the purposes of our experimental model. In this way, our experimental model could not verify if grape juice could promote some protection against muscle damage, lipid peroxidation and systemic inflammation. To better verify this hypothesis, it is necessary that the experimental session should occur with the athletes previously rested, but in this investigation, this was not possible, since it would require the laboratory conditions typical of studies of clinical level. Meanwhile, this study was done in the practical context of a national team in preparation for a world championship, so that the methodological aspect was lost, but gained in the context of the ecological validity of the study.

As limitations, this study was not able to verify the effect on the internal workload markers because of the methodological aspect that had to follow the training routine of the Brazilian selection of beach handball. The sample size could not be calculated because the sample was for convenience, based on the number of athletes called for national selection, on the other hand, the study gains in the practical application part of the training routine. The measurement of nitric oxide was done indirectly and our technique of serum measurement does not allow to determine which tissue produced this gas in the exercise/recovery session. Another limitation is that this study could not be double blind because has not been possible to make a placebo to grape juice, according to previous publications (Dalla Corte et al. 2013, Toscano et al. 2015, Miranda Neto et al. 2017). Recently, a placebo drink (Nova Aliança Winery, Flores da Cunha, Rio Grande do Sul, Brazil) was proposed, but the product tastes very light compared to whole juice, and was only available after the completion of this study.

CONCLUSION

Data from this study showed that a single dose supplementation with 400 ml of purple grape juice ingested immediately before (200 ml) and after (200 ml) a training session is able to promote the increase in nitric oxide production, due to increased levels of plasma nitrite, at the end of the recovery period. This increase in nitric oxide can improve the athlete’s performance, promoting antioxidant action, which is also beneficial for this population, although these ergogenic effects were not verified in the present study.

Acknowledgments

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