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Antioxidant and Neuroprotective Effect of Organic and Conventional White Grape Juices on Oxidative Stress Induced by Sodium Azide in Cerebral Cortex of Rats

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Author's contribution

This work was carried out in collaboration between all authors. Authors BRO, FDSM, NDSM, Camila Destri and ERDS performed the analyses of this study. Authors AQS, Caroline Dani and CF designed the study, performed the statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Diet plays an important role in the prevention of some diseases related to oxidative stress. Although the beneficial effects of a moderate intake of wine are well known, information about the antioxidant properties of grape juice is still limited. Therefore, the objective of this study was to investigate the *In vitro* neuroprotective effect of conventional and organic white grape juices (*Vitis labrusca*) on oxidative stress induced by sodium azide in cerebral cortex of 10-day-old-rats.
Study Design: Experimental study using an animal model.
Place and Duration of Study: Biochemistry Laboratory, Methodist University Center – IPA, Porto Alegre, RS, Brazil, between January and December 2012.
Methodology: Cerebral cortex was homogenized and preincubated for 30 minutes with

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40% (w/v) conventional or organic white grape juices, furthermore the homogenates were incubated for 1 hour with 5 mM sodium azide in the presence or absence of the juices. The assays of thiobarbituric acid reactive substances (TBARS), carbonyl, sulfhydryl, the activity of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) and the production of nitric oxide (NO) were performed in the homogenates.
Results: Sodium azide was able to enhance lipid peroxidation (TBARS) and carbonyl content and also to reduce the non-enzymatic antioxidants defenses (sulfhydryl) in cerebral cortex homogenates. Moreover, sodium azide inhibited the activity of CAT and SOD and enhanced NO levels. Conventional and organic white grape juices were able to prevent the effects caused by sodium azide in TBARS, carbonyl, sulfhydryl and CAT assays.
Conclusion: These results indicated that sodium azide induces oxidative stress in cerebral cortex of young rats and that conventional and organic white grape juices exhibit antioxidant properties capable to ameliorate the oxidative damage caused by this compound.

Keywords: Vitis labrusca; grapes; oxidative stress; neuroprotection.

1. INTRODUCTION

Oxidative stress represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess of reactive species can be an important mediator of damage to cell structures, and has been implicated in a number of human diseases [1-2]. On the other hand, several evidences indicate that a diet rich in fruits and vegetables reduces the risk of prevalent chronic health disorders such as cardiovascular and neurodegenetative diseases [3-5]. A number of mechanisms have been proposed for these associations, including the contribution of antioxidants from foods, such as vitamins C and E and polyphenols [4-6].

Some recently studies indicate that the consumption of grape juice offers health benefits by reducing the risk of diseases associated with oxidative stress [7-10]. In this context, grape juice is considered a very rich source of polyphenols [10-12], which has important *In vitro* [11] and *In vivo* antioxidant activity [8-9]. In South America, *Vitis labrusca* is the main specie used for wine and juice production [11]. In the last years an increasing interest in healthier and more environmentally friendly farming methods for different fruits, including grapes, has been observed. Nowadays, in Brazil, two types of grape juices are produced, conventional and organic. The conventional is produced from grapes that are treated with pesticides, while the organic is produced from grapes that do not receive any kind of chemical or genetic manipulation [13-14].

Considering that several studies have found a positive correlation between the incidence of chronic diseases with the oxidative stress [15-18], that dietary antioxidants have beneficial properties toward human health [19-20] and that there is only one study showing the *in vitro* benefits of *Vitis labrusca* white grape juice [11] the aim of this study was to demonstrate the *In vitro* antioxidant and neuroprotective effect of organic and conventional white grape juices on the oxidative stress induced by sodium azide in cerebral cortex of young rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Thiobarbituric acid (TBA), 2,4dinitrophenylhydrazine (DNPH), 5, 5' dithiobis (2-nitro benzoic acid) (DTNB), sulfanilamide, N-(1-naphthyl)ethylenediamine and sodium azide were purchased from SIGMA (St. Louis, MO, USA). All other reagents used were of analytical grade and were purchased from local suppliers.

2.2 Grape Juices

White grape juice samples used in this study were from *Vitis labrusca* grapes, Niagara variety. Organic grape juice was produced with grapes cultivated without pesticides, obtained from Cooperativa Aecia (Antonio Prado, Rio Grande do Sul, RS, Brazil) and certified by Rede de Agroecologia ECOVIDA. Conventional grape juice, produced with grapes cultivated using traditional methods, was obtained from Vínicola Perini (Farroupilha, Rio Grande do Sul, RS, Brazil). Validity periods were observed, and the same brands were used for the entire study. Grape juices were manufactured in 2011. The juices were manufactured by extraction, with a subsequent pressing in order to separate the pulp, and then submitted to pasteurization (at 85ºC), and immediately followed by bottling at 80 ºC.

2.3 Grape Juices Chemical Evaluation and Phenolic Compound Content

Total carbohydrates (g/100g) and density were determined according to AOAC International official methodologies [21]. Total phenolic content was measured using Singleton and Rossi's modification of Folin–Ciocalteau's colorimetric method [22].

2.4 Animals

Ten-day-old male or female Wistar rats (15–20 g) were obtained from our own breeding colony (n= 10). The animals were euthanazied by decapitation and the cerebral cortex was rapidly excised on a Petri dish placed on ice. The animals were maintained at approximately 22±2ºC, on a 12 h light/12 h dark cycle, with free access to water and food (54% carbohydrate, 4% fat, 4.5% fiber, 7% ashes and 10% moisture). The ''Principles of laboratory animal care'' (NIH publication no. 80–23, revised 1996) were followed in all experiments, and our research protocol was approved by the Ethical Committee for Animal Experimentation of Methodist University Center – IPA(14/2011). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.5 Tissue Preparation and Incubation

The cerebral cortex was homogenized in 1.5% KCl using a ground glass type Potter–Elvejhem homogenizer. The homogenates were centrifuged at 800 X g for 10 min at 4ºC, the pellet was discarded, and the supernatants were kept at -70ºC until the determinations were performed. The supernatants were preincubated at 30ºC for 30 minutes with 40% (w/v) of white conventional or organic grape juice, and afterwards sodium azide (5 mM) was added and the incubation continued for more 60 minutes.

2.6 Thiobarbituric Acid Reactive Substances (TBARS) Measurement

TBARS was used to determine lipid peroxidation. Trichloroacetic acid (10% w/v) was added to the homogenate to precipitate proteins and to acidify samples [23]. This mixture was then centrifuged (1000 X g, 3 minutes). The protein-free sample was extracted, and thiobarbituric acid (0.67% w/v) was added to the reaction medium. Tubes were placed in a water bath (100ºC) for 30 minutes. Absorbance was read at 535 nm in a spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments). Commercially available malondialdehyde was used as a standard. Results were expressed as mmol/mg of protein.

2.7 Carbonyl Assay

Carbonyl assay was used to determine oxidative damage to proteins. Homogenates were incubated with 2,4dinitrophenylhydrazine (DNPH 10 mmol/L) in 2.5 mol/L HCl solution for 1 hour at room temperature, in the dark. Samples were vortexed every 15 minutes. Then 20% TCA (w/v) solution was added in tube samples, left in ice for 10 minutes and centrifuged for 5 minutes at 1000 X g, to collect protein precipitates. Another wash was performed with 10% TCA. The pellet was washed 3 times with ethanol: ethyl acetate (1:1) (v/v). The final precipitate were dissolved in 6 mol/L guanidine hydrochloride solution, left for 10 minutes at 37ºC, and read at 360 nm [24] (T80 UV/VIS Spectrometer, PG Instruments).

2.8 Sulfhydryl Assay

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [25]. Briefly, 0.1 mM DTNB was added to 120 µL of the samples. This was followed by a 30 minutes incubation at room temperature in a dark room. Absorption was measured at 412 nm (T80 UV/VIS Spectrometer, PG Instruments). The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol/mg protein.

2.9 Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity, expressed as USOD/mg protein, was based on the inhibition of the ratio of autocatalytic adrenochrome formation at 480 nm (T80 UV/VIS Spectrometer, PG Instruments) [26]. Catalase(CAT) activity was determined by following the decrease in 240 nm absorption of hydrogen peroxide (H_2O_2) [27] (T80 UV/VIS Spectrometer, PG Instruments). It was expressed as UCAT/mg protein.

2.10 Nitric Oxide Production

Nitric oxide (NO) was determined by measuring the stable product nitrite through the colorimetric assay described by Hevel and Marletta [28]. In brief, the Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The reagent was added directly to the homogenates and incubated under reduced light at room temperature for 30 minutes. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–80 mM). Results were expressed as mmol/mg protein.

2.11 Protein Determination

Protein concentrations were determined by the method of Lowry et al. [29] using bovine serum albumin as standard.

2.12 Statistical Analysis

Grape juices composition was analyzed by student *t*-test and data from all other experiments were analyzed statistically by three-way ANOVA followed by the Tukey test. Values of $P<0.05$ were considered to be significant. All analyses were carried out using the Statistical Package for Social Sciences (SPSS) software (version 17.0).

3. RESULTS AND DISCUSSION

It is well described in the literature that oxidative damage is involved in the pathophysiology of the neurological disorders, such as epilepsy, Parkinson's and Alzheimer's diseases [17-18,30-31] and that deficits in brain function due to oxidative stress may be due to a decline in the endogenous antioxidant defense or to a higher production of reactive species [2]. Therefore, in the aim of the present study was to verify if the *In vitro* pretreatment with organic and conventional white grape juices was able to prevent the oxidative damage provoked by sodium azide in cerebral cortex of young rats.

The composition of organic and conventional white grape juices is shown in Table 1. It can be observed that there was no significant difference between density and total carbohydrates in both juices. However, the total phenolic content in organic white grape juice was significantly higher compared to the conventional white grape juice.

Table 1. Grape juices composition

	Conventional grape juice	Organic grape juice
Density	1.05±0.001	1.05±0.001
Total carbohydrates	14.0±1.2	13.07±0.1
Total phenolic content	7.37±0.15	22.41±0.16*

Total carbohydrates (g/100g); Total phenolic content (mg catechin/mL);
** P<0.05, student t-test*

Grapes are rich in phenolic compounds, such as resveratrol and flavonoids, which are found in higher concentrations in purples grapes [11,32]. Phenolic compounds are secondary metabolites produced and accumulated in plants, and changes in its defenses, along with other factors, might result in different concentrations of these compounds. The organic farming is widely practiced throughout the world, and it is characterized by not using chemicals, pesticides or synthetic fertilizers. Without the protection offered by synthetic pesticides plants are more susceptible to the action of pathogens, and produce more phenolic compounds [33-34]. In this context, here we observed an enhanced in total phenolic compounds in organic white grape juice compared to the conventional one (Table 1). Our results are in line with previous studies that show that the organic products are richer in phenolic content [10-11,35-36].

In the present study we used sodium azide as an experimental model for oxidative damage. This compound acts by inhibiting complex IV (cytochrome oxidase) in the electron transport

chain, which may causes electrons leakage and thus resulting in oxidative stress [37]. Therefore, next, we evaluated the oxidative damage provoked by sodium azide and the possible antioxidant and neuroprotecitve effect of the grape juices in homogenates of cerebral cortex of rats (Figs. 1, 2, 3 and 4). Sodium azide induced an increase in lipid peroxidation (TBARS) (Fig. 1A) and protein oxidation (carbonyl) (Fig. 1B) in cerebral cortex of rats and both conventional and the organic grape juices were able to prevent this increase (Fig. 1).

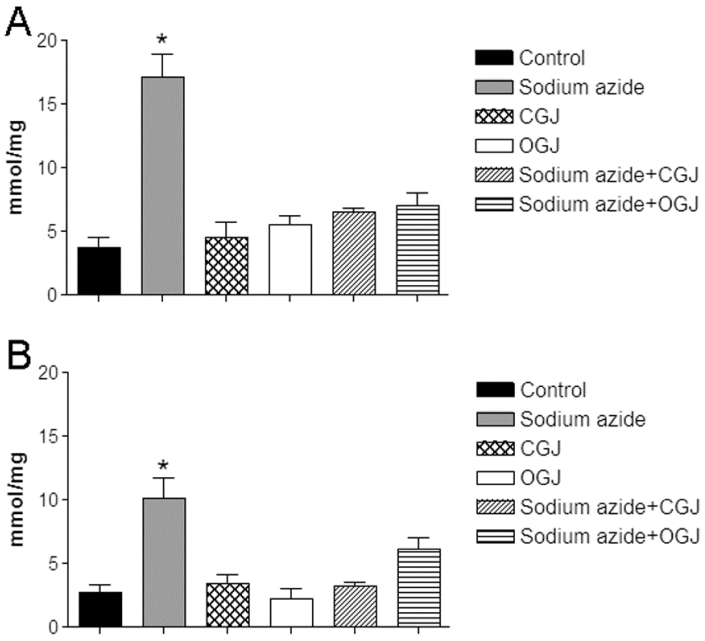

Fig. 1. Effect of sodium azide, conventional and organic white grape juices on thiobarbituric acid reactive substances (TBARS) (A) and protein carbonyls (B) in the cerebral cortex of rats

Data are expressed as mean ± SD and were statistically analyzed by three-way ANOVA followed by Tukey test.

**P<0.05, from other groups. OGJ = organic grape juice, CGJ = conventional grape juice. N=10*

This evidence is in line with another study showing that purple grape juice from *Vitis labrusca* prevented the oxidative damage caused by the toxic agent carbon tetrachloride (CCl_4) in brain structures, plasma and liver of adult rats [8-9]. Our group also demonstrated that grape seed extract and grape leaves extract were capable to prevent the oxidative damage to lipids and proteins provoked by hydrogen peroxide (H_2O_2) in central and peripheral tissues [38-40]. It is important to emphasize that an increase in oxidative damage is closed linked to several pathological disorders such as rheumatoid arthritis, Alzheimer's disease, Parkinson's disease, seizures disorders and atherosclerosis [17-18,30-31,41].

We also verified the effect of sodimazide on the non-enzymatic antioxidant defenses by measuring protein sulfhydryl groups. Fig. 2 shows that sodium azide reduced the sulfhydryl groups in the cerebral cortex of rats and both grape juices prevented this effect. It also can be observed that organic grape juice was capable to increase the sulfhydryl content compared to control group even in the presence of sodium azide (Fig. 2).

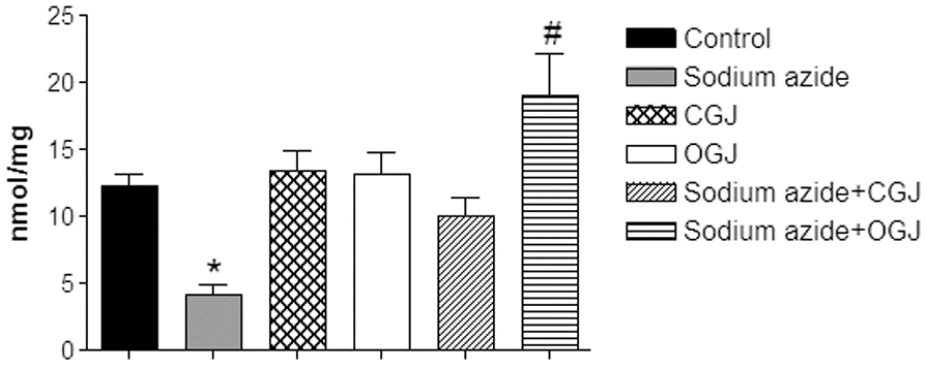

Fig. 2. Effect of sodium azide, conventional and organic white grape juices on sulfhydryl groups in the cerebral cortex of rats

Data are expressed as mean ± SD and were statistically analyzed by three-way ANOVA followed by Tukey test.

**P<0.001, from other groups; #P<0.01 from control. OGJ = organic grape juice, CGJ = conventional grape juice. N=10*

Thiols (SH) are a class of organic sulfur derivatives characterized by the presence of sulfhydryl residues. In biological systems, SH have numerous functions, including a central role in coordinating the non-enzymatic antioxidant defense network [42]. Reduced concentration of SH-groups has been reported in several chronic diseases, such as diabetes, and in aging process [43-44]. At this moment, we could postulate that this greater protection of the organic grape juice could be due to the higher concentration of polyphenols in this juice, as compared to the conventional grape juice (Table 1). The beneficial effect of polyphenols is also described by other researches. Pandey and Rizvi [45] showed that tert-butyl hydroperoxide (t-BHP) reduced the SH-groups and that resveratrol was capable to enhance this content in human plasma. This increase in non-antioxidant defenses is also demonstrated in others grape derivate products, e.g. grape seed extract and grape leaves extracts in several tissues of rats [38-40].

The antioxidant enzymes SOD and CAT are responsible for the enzymatic antioxidant defenses and represent the first barrier against reactive species [46]. The enzyme SOD acts by dismutation of superoxide anion ($O2^{-\bullet}$) into oxygen (O_2) and H_2O_2. The enzyme CAT catalyzes the reduction of H_2O_2 to water and O_2. Therefore, we also investigated the effect of sodium azide on the enzymatic antioxidant defenses by measuring CAT and SOD activities. Sodium azide decreased the enzymatic antioxidant defenses in the cerebral cortex of rats (Fig. 3). Conventional and organic grape juices were able to prevent CAT inhibition (Fig. 3A). However, both grape juices also decreased SOD activity and were unable to prevent the reduction caused by sodium azide (Fig. 3B).

These results are in line with Oliboni et al. [39] and Rodrigues et al. [10], that observed a prevention of CAT activity but not for SOD activity in rats tissues using grape derivatives (grape leaves extract and purple grape juice, respectively). On the other hand, some studies showed that polyphenols were capable to attenuate the decrease in the activities of SOD and CAT [47-49].

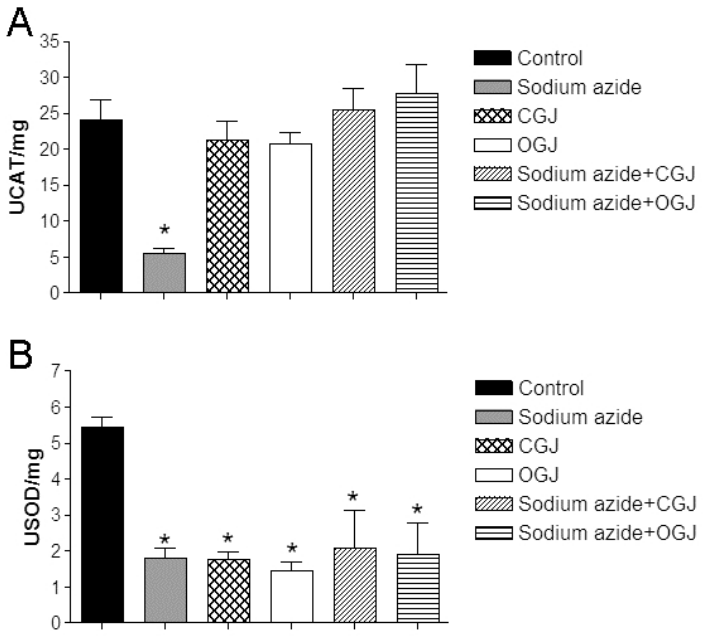

Fig. 3. Effect of sodium azide, conventional and organic white grape juices on the activity of the enzyme catalase (CAT) (A) and activity of superoxide dismutase (SOD) in the cerebral cortex of rats

Data are expressed as mean ± SD and were statistically analyzed by three-way ANOVA followed by Tukey test.

** P<0.05, from control. OGJ = organic grape juice, CGJ = conventional grape juice. N=10*

We also verified here that sodium azide caused an increase in NO production. However, both white grape juices were not able to ameliorate this effect (Fig. 4). This data might be related to the fact that polyphenols present in grape products (e.g. dealcholized red wine and wine) are capable to increase NO levels [50-51].

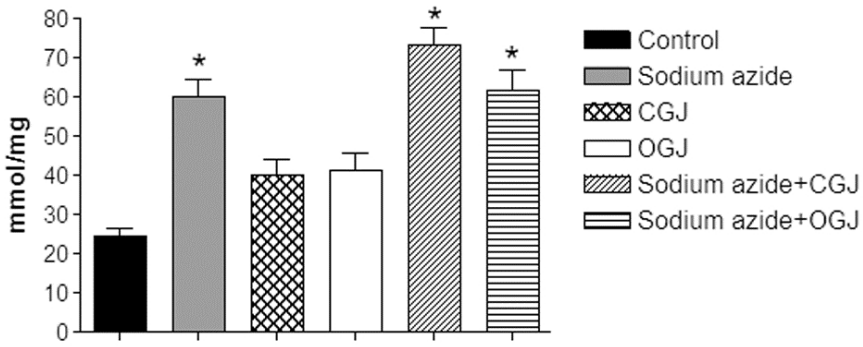

Fig. 4. Effect of sodium azide, conventional and organic white grape juices on nitric oxide production in the cerebral cortex of rats

Data are expressed as mean ± SD and were statistically analyzed by three-way ANOVA followed by Tukey test.

** P<0.05, from control. OGJ = organic grape juice, CGJ = conventional grape juice. N=10*

Taken together, we demonstrated that the white grape juices, conventional and organic, were not able to prevent the inhibition of SOD activity and the enhanced of NO levels provoked by sodium azide. Therefore, NO could react with $O2^{-\bullet}$ forming peroxynitrite ($ONOO^{-}$), the most harmful NO derivative, that can modify biomolecules, including DNA, lipids, and proteins [52-53].

4. CONCLUSION

In conclusion, the data presented here showed for the first time that the white grape juices (organic and conventional) are able to ameliorate the *In vitro* oxidative damage caused by sodium azide in the cerebral cortex of rats. Moreover, it is important to emphasize that in this study we observed antioxidant properties of grape juices based on their capability of scavenging free radicals *In vitro* and it is not established that this capability exerts a beneficial physiological effect in humans as required by Regulation (EC) No 1924/2006 and the European Food Safety Authority (EFSA) guidance on scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health [54].

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Ethical Committee for Animal Experimentation of Methodist University Center – IPA (14/2011).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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