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Protective Effects of Purple Grape Juice on Carbon Tetrachloride-Induced Oxidative Stress in Brains of Adult Wistar Rats

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ABSTRACT The antioxidant properties of purple grape juice, organic and conventional, in brain tissues are not well known. In this study our objective was to evaluate the antioxidant activity in substantia nigra and striatum of rats chronically treated with organic or conventional purple grape juice and to correlate the results obtained with the polyphenol content (total polyphenolic content, resveratrol, and anthocyanins [malvidin, delphinidin, peonidin, and cyanidin]). We observed that CCl_4 damage decreased significantly in the grape juice-treated groups when compared with the control group. In the grape juice-treated groups we further observed a decrease of lipid (thiobarbituric acid-reactive substances assay) and protein (carbonyl) peroxidation, as well as a significant antioxidant protection through the increase of enzyme activity. Antioxidant activities were significantly correlated with polyphenol content. These findings demonstrated that both grape juices have potent antioxidant properties and these activities could be at least attributed to the high phenolic content present in these juices.

KEY WORDS: • ***antioxidant*** • ***grape juice*** • ***neuroprotection*** • ***oxidative stress*** • ***phenolic content***

INTRODUCTION

Nowadays several studies have shown that a high consumption of vegetables and fruits is consistently associated with a low risk of oxidative stress-induced diseases. Polyphenols are the main compounds believed to be responsible for this protection. These classes of compounds are found in many fruits, like grapes and its products.[1] Grape juice is a very rich source of polyphenols, such as flavonoids and anthocyanides, and nonflavonoids, such as resveratrol.[2]

CCl_4-induced toxicity is a well-characterized murine model for oxidative damage *in vivo*. The toxicity of CCl_4 results from its reductive dehalogenation by the cytochrome P450 enzyme system into a trichloromethyl free radical, which readily interacts with molecular oxygen to form the trichloromethyl peroxyl radical.[3]

Several studies have demonstrated that the liver is not the only target organ of CCl_4. CCl_4 has been reported to cause lipid peroxidation in other organs such as kidney, heart, and brain.[3,4] In the present study, levels of thiobarbituric acid-reactive substances (TBARS) and carbonyl proteins and activities of antioxidant enzymes were quantified in brain structures to examine the antioxidant activity with purple grape juice pretreatment.

The substantia nigra and the striatum, components of the dopaminergic pathway, play an important role in the regulation of movements and of some cognitive functions. The high metabolic rate and the oxidative degradation of dopamine by the mitochondrial monoamine oxidase in these structures contribute to the oxidative damage in biomolecules. Indeed, oxidative stress is involved in some pathologies that affect the nigrostriatal axis.[5]

There is an increasing interest in healthier and more environmentally friendly production methods for different fruits. Nowadays organic production is not one cultivation method, but many, characterized by restrictions against the use of synthetic pesticides and synthetic fertilizers, although the detailed regulations of what can be called organically cultivated vary.[6,7] Nevertheless, consumers today buy products that are labeled "organically cultivated" in stores, and many expect the quality to be superior to that of conventionally cultivated products in terms of contents that make the products healthier.[8] There are some studies that showed some differences in antioxidant activities and in the phenolic content in fruit juices obtained from organic and conventional procedures, but until now these studies are inconclusive.[6,8]

Although there are many brain diseases caused by free radicals, like Parkinson's disease, we did not find consistent data in literature suggesting a relationship between grape

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juice intake and antioxidant protection to the central nervous system. One study showed that different Concord grape juice concentrations (10% and 50%) were beneficial in reversing the course of neuronal and behavioral aging.[9] Given these considerations, the aim of the present study was to investigate the beneficial effects of organic and conventional purple grape juices in reducing the oxidative stress in the striatum and the substantia nigra isolated from 60-day-old Wistar rat brains.

MATERIALS AND METHODS

Grapes and grape juices

The grape juice samples used in this work were made with grapes of the *Vitis labrusca* Bordo variety. The organic juices were obtained from the Cooperativa Aecia (Antonio Prado, RS, Brazil) and were certified by ECOVIDA, while the conventional juices were obtained from Vinhos Monte Reale (Flores da Cunha, RS, Brazil). Throughout the tests, we observed the expiry dates of the juices and always used the same trademarks.

Phenolic compounds

Total phenolic content was measured using the modification of Singleton *et al.*[10] of the Folin-Ciocalteau colorimetric method. High-performance liquid chromatography (HPLC) analysis was used in order to quantify the presence of individual phenolic compounds. Before the HPLC analysis, 5 mL of each sample was filtered through a cellulose membrane (diameter, 0.20 mm). The equipment used in the analysis consisted of an LC-DAD Series 1100 liquid chromatographic system (Hewlett-Packard, Palo Alto, CA) with a diode array detector system.

Resveratrol analysis. In order to quantify the *trans*-resveratrol compound, we used a mobile phase of ultrapure water and acetonitrile (75:25 vol/vol) (pH 3.0) in a constant flow of 1.0 mL/minute for 20 minutes in a controlled-temperature room at 20°C. The peak was detected at 306 nm, and the amount of sample injected was 20 μL.[11]

Anthocyanin analysis. In order to determine cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside, we used a mobile phase with solvents A (ultrapure water, formic acid, and acetonitrile) and B (ultrapure water, formic acid, and acetonitrile) in a constant flow of 0.8 mL/minute in a controlled-temperature room at 25°C. The peak was detected at 518 nm, and the amount of sample injected was 50 μL.[12]

Animals

Twenty-four male Wistar rats (60 days old, weighing 200 $\pm$ 50 g) from our breeding colony were used in the experiments. The animals were handled under standard laboratory conditions of a 12-hour light/dark cycle and fixed temperature (25 $\pm$ 2°C). Food and water were available *ad libitum*. All experimental procedures were performed in accordance with the U.S. National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* with the approval of the local ethics committee.

Chronic intake

The animals were randomly allocated to one of the three experimental groups (n = 8): group 1 served as the control and received saline, and groups 2 and 3 were given purple grape juice (conventional and organic, respectively). The doses of purple grape juice were determined by calculating the daily amount of juice consumed on average by a 70-kg human male. As a reference, we used a study with humans who received 480 mL/day.[13] The amount of juice was administered to the rats according to their body weight. We gave 7 μL of grape juice/g of body weight, twice a day; however, during the experiment the amount varied. On day 30, half of the animals received a single intraperitoneal dose of CCl_4 (3 mL/kg). The animals that received CCl_4 (positive control) or animals that received only oil (vehicle) (negative control) were killed 6 hours later by decapitation. Brain structures were isolated and stored at -70°C until analysis.

Oxidative stress parameters

As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction, which is widely adopted as a sensitive method for measurement of lipid peroxidation, as previously described.[14]

The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhydrazine, as previously described.[15]

Antioxidant enzyme assays were performed in tissue homogenates, as previously described.[3] Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm.[16] Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline autooxidation, as previously described.[17]

Statistical analyses

Statistics were calculated by means of analysis of variance and Tukey's test using the SPSS (Chicago, IL) version 12.0 package. All tests were performed in duplicate. Pearson's correlation coefficient was used to test correlation between polyphenol content and the assays.

RESULTS

Table 1 presents additional information about the content of phenolic compounds in the two types of purple grape juice used in this study. There is a statistical difference in the content of total phenolic compounds ($P < .05$) between both juices, especially in the resveratrol amount; the organic purple grape juice had higher amounts in both parameters. It is possible to observe an important difference between these

TABLE 1. TOTAL PHENOLIC CONTENT AND LEVELS OF RESVERATROL AND ANTHOCYANINS (CYANIDIN, DELPHINIDIN, PEONIDIN, AND MALVIDIN) IN ORGANIC AND CONVENTIONAL GRAPE JUICES

	Grape juice	
	Organic	*Conventional*
Total phenolic compounds (mg of catechin/mL)	262.50 ± 0.70*	119.59 ± 3.53
Resveratrol amount (ppm)	0.213 ± 0.005*	0.075 ± 0.010
Cyanidin (ppm)	11.79 ± 0.42*	0.76 ± 0.04
Delphinidin (ppm)	26.30 ± 1.15*	4.10 ± 0.40
Malvidin (ppm)	232.46 ± 4.25*	95.26 ± 1.95

Data are mean ± SD values.
*Statistically difference between the two grape juices ($P < .05$).

two grape juices in content of anthocyanins (malvidin, cianidin, delphinidin, and peonidin): the organic juice was richer than the conventional one (Table 1).

CCl_4 induced an increase in lipid peroxidation in the striatum and substantia nigra when compared to the control group ($P < .05$). The chronic treatment with both grape juices did not induce significant differences in the striatum when compared to the control group, but we observed a significant reduction in TBARS in the substantia nigra of isolated organic juice-treated rats ($P < .05$). Both grape juices had a protective effect when comparing the CCl_4-induced groups (Fig. 1).

Concerning protein peroxidation damage (carbonyl assay), there is a significant difference between the CCl_4 and vehicle groups, in which CCl_4 increased the protein oxidative level as expected (Fig. 2). However, after the intake of organic grape juice, carbonyl levels showed an important and significant attenuation in the striatum when compared with the negative (vehicle) and positive (CCl_4) controls (Fig. 2A). This decrease showed a positive correlation with all phenolic compounds: resveratrol ($r = 0.786$), malvidin ($r = 0.796$), delphinidin ($r = 0.796$), peonidin ($r = 0.786$), cianidin ($r = 0.786$), and total phenolic content ($r = 0.693$) (all $P < .05$).. In the substantia nigra both organic and conventional grape juices were capable of reducing the carbonyl levels when compared to CCl_4-induced groups, but the conventional juice showed a better performance than the organic juice (Fig. 2B). In these groups the decrease in carbonyl levels showed also an important correlation with the grape juice phenolic content: resveratrol ($r = 0.493$), malvidin ($r = 0.534$), delphinidin ($r = 0.493$), peonidin ($r = 0.453$), cianidin ($r = 0.493$), and total phenolic content ($r = 0.530$) (all $P < .05$).

Both grape juice-treated groups showed higher CAT activity ($P < .05$) in the striatum as compared to the negative and positive controls. In this same structure we observed a significant increase in the positive control (CCl_4) when compared with the negative control (Fig. 3A). CAT activity was increased in both grape juice groups when compared with the positive control group in striatum. In this tissue, there was a positive correlation between CAT activity and total polyphenol content ($r = 0.539$; $P < .01$), resveratrol ($r = 0.552$; $P < .01$), cyanidin ($r = 0.450$; $P < .01$), delphinidin ($r = 0.476$; $P < .05$), peonidin ($r = 0.538$; $P < .01$), and malvidin ($r = 0.533$; $P < .01$). Because of this positive correlation, the increase of CAT activity could be explained, in part, by the presence of these compounds. Furthermore, the positive control group showed a significant decrease ($P <$

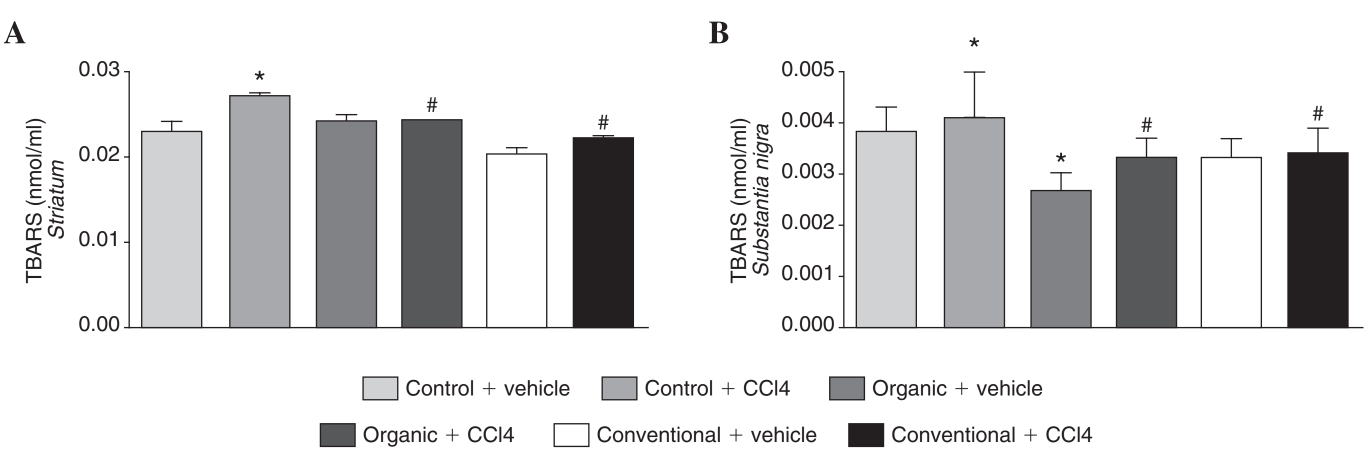

FIG. 1. TBARS level in (**A**) striatum and (**B**) substantia nigra with different grape juice treatments. *$P < .05$ compared to control + vehicle; #$P < .05$ comparing the grape juice treatments to control + CCl_4.

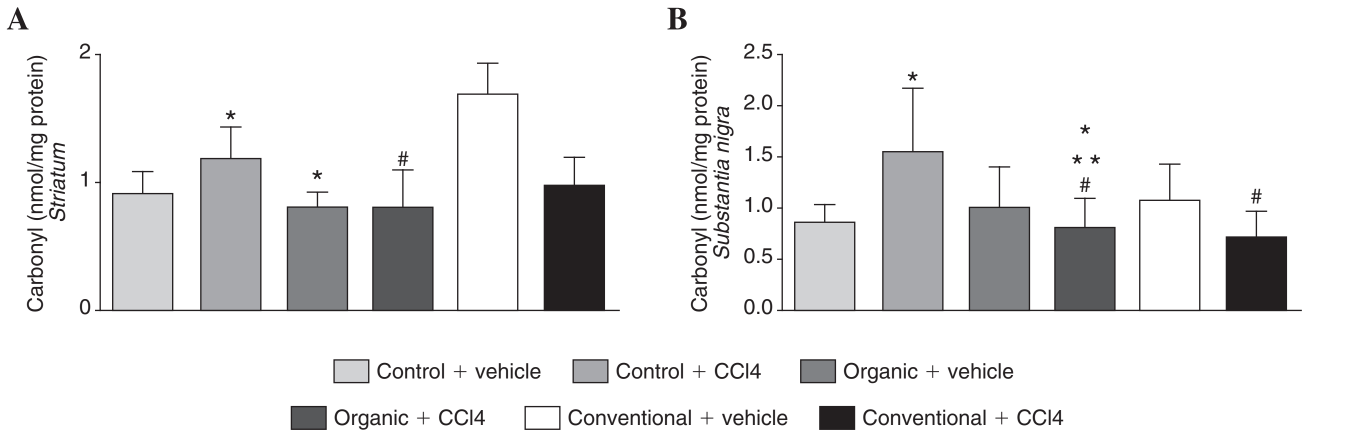

FIG. 2. Protein peroxidation level (carbonyl assay) in (**A**) striatum and (**B**) substantia nigra with different grape juice treatments. $*P < .05$ compared to control + vehicle; $**P < .05$ comparing the organic and conventional grape juice treatments; $^{\#}P < .05$ comparing the grape juice treatments to control + CCl_4.

.005) in the substantia nigra when compared with the negative control (Fig. 3B). A positive correlation was observed when for CAT activity with total phenolic and peonidin content ($r = 0.437$ and 0.434, respectively; $P < .05$).

When analyzing the SOD activity it was observed that the organic grape juice combined with CCl_4 increased the activity of this enzyme in the striatum when compared with the positive control, showing a positive correlation with resveratrol content ($r = 0.633$; $P < .05$) (Fig. 4A). In the substantia nigra the organic grape juice group presented the highest values of SOD activity when compared with both controls, negative and positive (Fig. 4B). A positive correlation was observed between SOD activity and contents of the phenolic compounds resveratrol ($r = 0.693$; $P < .01$), cyanidin ($r = 0.689$; $P < .05$), peonidin ($r = 0.682$; $P < .01$), delphinidin ($r = 0.695$; $P < .01$), and malvidin ($r = 0.687$; $P < .01$) and total phenolic content ($r = 0.681$; $P < .05$), showing an important action of these compounds in increasing this enzyme activity after CCl_4 exposure.

We observed a significant decrease of the SOD/CAT ratio in the striatum and the substantia nigra in both chronic grape juice intake groups when compared with the positive control; however, the organic juice was able to reduce this ratio in the striatum more than the conventional juice (Fig. 5). Indeed, in both grape juice-treated groups the SOD/CAT ratio showed lowest levels in the striatum and substantia nigra when compared with the negative control (vehicle), but the organic grape juice reduced this level more significantly in the striatum ($P < .005$) (Fig. 5). This high reduction property of organic grape juice could be explained, at least in part, as being due to the rich phenolic content analyzed in this juice, which in striatum and substantia nigra showed a negative correlation with the SOD/CAT ratio. In striatum this correlation was observed with resveratrol ($r = -0.577$; $P < .01$), cyanidin ($r = -0.587$; $P < .01$), peonidin ($r = -0.591$; $P < .01$), delphinidin ($r = -0.501$; $P < .05$), and malvidin ($r = -0.587$; $P < .01$).

DISCUSSION

Organisms can suffer oxidative damage, yet the animal brain is often said to be especially sensitive; one reason is its high oxygen consumption and high lipid content. The

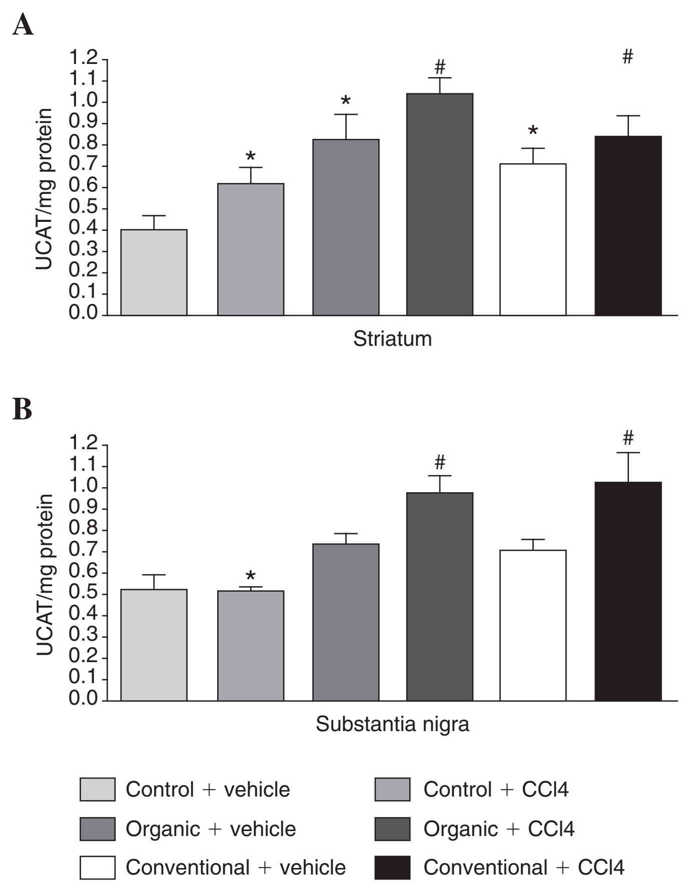

FIG. 3. CAT activity level (in units [U] of enzyme activity) in (**A**) striatum and (**B**) substantia nigra with different grape juice treatments. $*P < .05$ compared to control + vehicle; $^{\#}P < .05$ comparing the grape juice treatments to control + CCl_4.

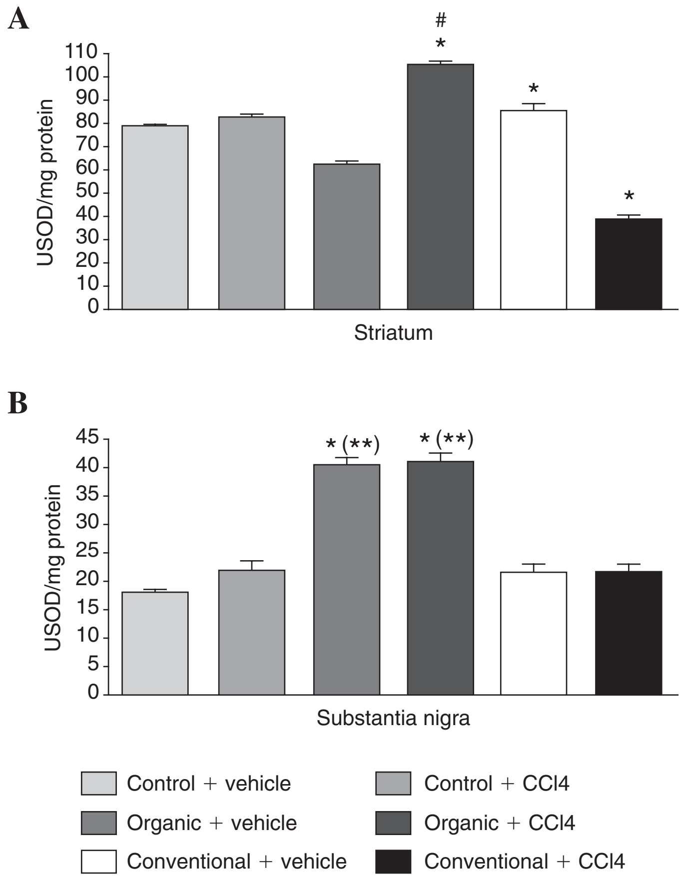

FIG. 4. SOD activity level (in units [U] of enzyme activity) in (**A**) striatum and (**B**) substantia nigra with different grape juice treatments. $^{*}P < .05$ compared to control + vehicle; $^{**}P < .05$ comparing the organic and conventional grape juice treatments; $^{\#}P < .05$ comparing the grape juice treatments to control + CCl_4.

present study is the first one to show that purple grape juice can reduce oxidative stress in structures of the nervous system, such as the substantia nigra and the striatum. In another study, the intake of juice obtained from the Concord grape, a variety of purple grape, was able to enhance motor performance and dopamine release.[9]

In our study we observed that chronic grape juice intake reduced lipid peroxidation and oxidative protein damage after treatment with CCl_4, known as an oxidative stressor.[3] The reduction of the lipid peroxidation level in brain structures was observed in other studies with rats that received grape seed extract.[18] In the present study, the reduction observed could be explained, at least in part, by the phenolic content—of which grape products show an important amount.

This fact could be observed in our study: positive correlations were observed between the phenolic content and the reduction of the lipid peroxidation and protein oxidation (carbonyl) inducted by CCl_4. CCl_4-induced toxicity is a well-characterized murine model for oxidative damage *in vivo*, because the radicals produced by this agent are able to attack proteins and lipids or still abstract hydrogen atoms from an unsaturated lipid, leading to membrane lipid peroxidation, cellular dysfunction, and finally cell necrosis.[19] Against these damages the organisms have important antioxidant defenses, such as SOD and CAT enzymes; however, other nonenzymatic compounds can reduce this damage, such as the polyphenol compounds. Many biological activities are attributed to phenolic compounds, *e.g.*, antioxidant, anti-inflammatory, and anti-oncogenic.[20] Data from a recent study suggest that *Vitis vinifera* can be used as a chemopreventive agent against oxidative stress and carcinogenesis, mainly because of their phenolic compounds.[21]

Park *et al.*[13] showed in their study that purple grape juice was capable of reducing DNA damage, as evidenced by the Comet assay. It has already been reported that the compounds present in grape juice can inhibit (1) platelet activity,[22] (2) low-density lipoprotein oxidation and oxidative damage to DNA,[13] and (3) coronary disease and atherosclerosis.[1]

Phenolic compounds are secondary metabolites produced and accumulated in plant tissues. Nowadays organic farming is a widely utilized small-scale practice, in which no chemical substances like pesticides or artificial fertilizers to

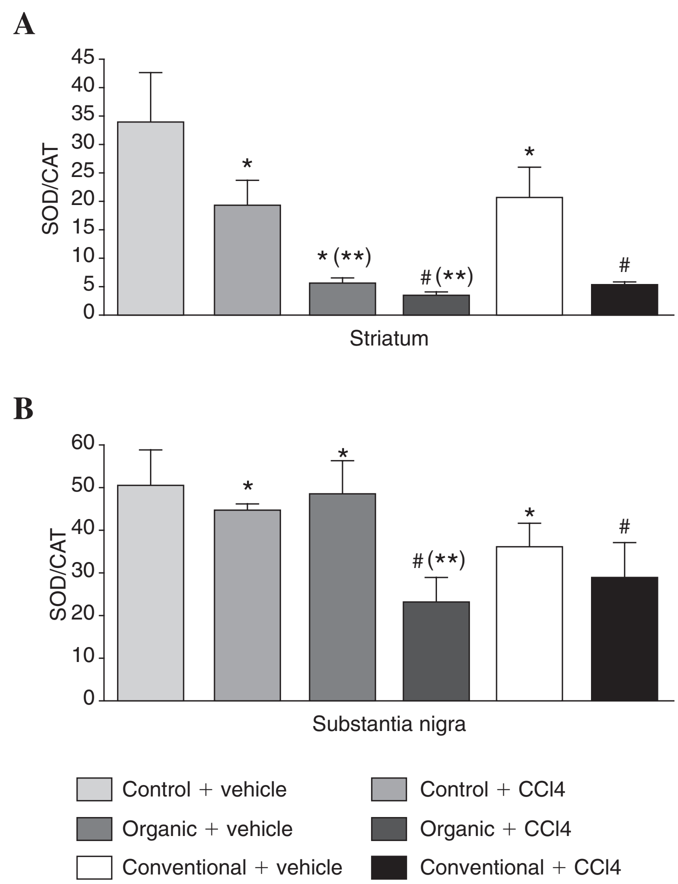

FIG. 5. SOD/CAT ratio in (**A**) striatum and (**B**) substantia nigra with different grape juice treatments. $^{*}P < .05$ compared to control + vehicle; $^{**}P < .05$ comparing the organic and conventional grape juice treatments; $^{\#}P < .05$ comparing the grape juice treatments to control + CCl_4.

promote plant growth are used. Since pesticides are not used, plants are more susceptible to the action of phytopathogenic organisms, resulting in the production of larger amounts of phenolic compounds.[20] We observed that depending on which agricultural practice is chosen (organic vs. conventional), it will result in different amounts of resveratrol, anthocyanins, and tannins, with the organic practice being richest in these compounds (Table 1).

The brain has antioxidant defenses that could act against free radicals, but with aging these defenses decrease. As defenses one can cite enzymes such as SOD and CAT. SOD plays a key role in detoxifying superoxide anions, which otherwise damage the cell membranes and macromolecules.[23] Clinical studies demonstrated a reduction of SOD activity in parkinsonism associated with other neurodegenerative disorders, showing the key role played by this enzyme in fighting free radicals produced in the brain.[24,25]

In the present study, CAT activity was increased after chronic intake of purple grape juice, especially in the organic juice group, suggesting that a high content in phenolic compounds is capable of increasing the activity of the enzymatic antioxidant defenses. SOD activity was significantly increased in the purple grape juice chronic intake groups, once more suggesting that the juice is capable of increasing the activity of the enzymatic antioxidant defenses. Indeed, the organic grape juice group showed the highest increase, a fact that could be explained by the richer content of total phenolics and higher levels of resveratrol and anthocyanins found in the organic juice. Recently, a study reported that resveratrol was capable of reducing the lipid peroxidation and increasing activities of the antioxidant enzymes SOD and CAT.[26] Furthermore, this increased enzymatic activity was also observed in other studies that reported higher SOD and CAT activities in brain regions of rats treated with grape seed extracts.[18] Balu *et al.*[18] attributed this increase to the free radical quenching action of the dihydroxyl (catechol) structure in the B-ring of proanthocyanin present in grape seeds.

We could also observe that after chronic intake of grape juice the SOD/CAT ratio was lower in this group than in the control. In the striatum, the organic grape juice group showed the lowest ratio level when compared with the negative and positive control groups. The SOD/CAT ratio is a very important parameter because an imbalance between these two enzymes of the antioxidant defenses may induce an increase in oxidative stress level and thus to the development of many diseases associated with it.[27] SOD activity leads to the production of hydrogen peroxide, which reacts with iron to generate hydroxyl radicals via the Fenton reaction, which in turn are thought to be the most toxic oxygen molecules *in vivo.*[28] CAT could clean up an excess of hydrogen peroxide, thus diminishing its oxidative effects. Our results showed that treatment with grape juice can induce an increase in CAT and SOD activity and reduce the SOD/CAT ratio, suggesting an important antioxidant activity of grape juice, and with no reactive species being produced in excess, proving a grape juice health benefit shown in another study.[29]

In conclusion, the results of the present study suggest that grape juice intake enhances antioxidant status in rats by reducing the levels of lipid peroxidation end products, whose accumulation would otherwise play a key role in brain aging, and reduces the incidence of brain diseases, such as Parkinson's, Alzheimer's, and other diseases. Further studies are needed to obtain a better understanding of the molecular mechanism by which chronic grape juice intake may modulate CAT and SOD activities.

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