Neuroprotective Effect of Organic and Conventional White Grape Juice against Carbon Tetrachloride Damage in Different Brain Areas of Rats

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Abstract

The consumption of nutrients containing phenolic compounds has been reported due to the benefits they produce on human health. Therefore, the objective of this study was to investigate the antioxidant and neuroprotective effect of the administration of organic (OGJ) and conventional (CGJ) white grape juices from Niagara variety on the oxidative stress in cerebral cortex, hippocampus and cerebellum after the treatment with carbon tetrachloride (CCl4) as well as on some biochemical parameters in serum of rats. Adult male rats (~300g; n=6-8/group) were orally treated (gavage) with 7μL/g of OGJ, CGJ or water, for a period of 14 days. On the 15th day it was administered CCl4 (3.0mL/kg). After 4h the animals were euthanized and the cerebral cortex, hippocampus and cerebellum were dissected and used for the analysis of oxidative stress parameters. We observed that CCl4 enhanced lipid peroxidation (TBARS) and protein damage (carbonyl), reduced the non-enzymatic antioxidants defenses (sulfhydryl), and changed the activity of the enzymatic antioxidants defenses catalase (CAT), Superoxide Dismutase (SOD) in the brain of rats. CCl4 also enhanced glucose, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Gamma-Glutamyl (GGT) and decreased catalase (CAT), Superoxide Dismutase (SOD) in the brain of rats. CCl4 also enhanced glucose, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Gamma-Glutamyl (GGT) and decreased Catalase (CAT), Superoxide Dismutase (SOD) in the brain of rats.

Introduction

Oxidative stress is defined as an imbalance between free radicals and reactive species production, and antioxidant defense mechanisms [1-3]. Several studies have shown that oxidative stress is closely linked to the pathogenesis of many diseases, such as: Alzheimer’s disease, Parkinson’s disease, diabetes, hypertension and cardiovascular diseases, multiple sclerosis and certain types of cancers [1,4,5]. However, reactive species also are very important for normal metabolism, signal transduction and regulation of cellular functions. Therefore, each cell should maintain the homeostasis between the pro-oxidant and antioxidant species [6].

Antioxidants are vital substances, which possess the ability to protect from the damage caused by oxidative stress [7]. It is well described that a number of dietary sources such as fruits and vegetables have the ability to scavenge free radicals or reactive species and consequently act as antioxidants [8,9]. In this context, grapes which are rich in phenolic compounds, such as flavonoids (catechin, epicatechin, quercetin, anthocyanins and procyanidins), and resveratrol have antioxidant properties [10].

In Brazil, Serra Gaucha, located in the northeastern state of Rio Grande do Sul, is the largest wine-growing region of the country with about 40 hectares of vineyards [11]. Currently, two distinct classes of grape juices are produced, the conventional, made from grapes that have been treated with pesticides, such as herbicides and fungicides, and the organic, which is produced from grapes that have not received any kind of chemical or genetic manipulation [12,13].

Considering that it has been already reported that purple grape juice can afford protection against platelet aggregation, Low-Density Lipoprotein (LDL) oxidation, oxidative damage to Deoxyribonucleic Acid (DNA), coronary diseases, atherosclerosis and brain oxidative damage caused by a convulsing drug (pentylenetetrazole), carbon tetrachloride (CCl4) and high-fat diet consumption [14-19] and that there are only few studies that have demonstrated the beneficial potential of white grape juices [12,20] the objective this study was to verify the antioxidant and neuroprotective effect of organic (OGJ) and conventional (CGJ) white grape juices on the oxidative stress induced by CCl4 in different brain areas and on some biochemical parameters in serum of rats.
Material and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for thiobarbituric acid, which was from Merck (Darmstadt, Germany).

Grape juices

White grape juice samples used in this study were from Vitis labruscag grapes, Niagara variety. OGJ was produced with grapes cultivated without pesticides. It was obtained from Indústria e Comércio de Doces e Conservas Carraro Ltda (Monte Alegre dos Campos, RS, Brazil) and certified by Rede de Agroecologia ECOVIDA. CGJ, produced with grapes cultivated using traditional methods, was obtained from Vinícola 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 by extraction, with a subsequent pressing in order to separate the pulp, then submitted to pasteurization (at 85ºC), and immediately followed by bottling at 80ºC.

Grape juices chemical evaluation and phenolic compound content

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

Animals

Forty adult male Wistar rats (~300g; 90-days-old) were obtained from our own breeding colony. They were maintained at 22 ± 2°C, on a 12-h light/12-h dark cycle, with free access to food and water. 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 the Centro Universitário Metodista - IPA, Porto Alegre, Brazil. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Treatment

The animals were randomly divided into three experimental groups. Group 1 received water (7μL/g); Group 2 received CGJ (7μL/g); Group 3 received OGJ (7μL/g). All animals were orally (gavage) administered with a single daily dose of the juices or water during 14 days. On the 15th day half of the animals in each group received a single intraperitoneal injection of CCl4, in a dose of 3.0mL/kg and the other half of the animals received only vehicle (mineral oil) [17]. After 4 h the animals were euthanized by decapitation (7μL/g); Group 3 received OGJ (7μL/g). All animals were orally (gavage) administered with a single daily dose of the juices or water during 14 days. On the 15th day half of the animals in each group received a single intraperitoneal injection of CCl4, in a dose of 3.0mL/kg and the other half of the animals received only vehicle (mineral oil) [17]. After 4 h the animals were euthanized by decapitation and the cerebral cortex, the hippocampus and the cerebellum were dissected and kept chilled until homogenization. The trunk blood was collected in tubes without any anticoagulant and it was used for the determination of the biochemical parameters.

Biochemical parameters determination

Serum was obtained by centrifugation of the trunk blood at 1000 × g for 10 min (hemolysed serum was discarded). Glucose, triglycerides, total cholesterol and High Density Lipoprotein (HDL) were used as biochemical markers. Hepatic function was analyzed using Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma-Glutamyl (GGT) activities as markers of toxicity. Renal function was analyzed by determining urea and creatinine. All assays were carried out using commercial kits (Labtest, Diagnostica S.A., Minas Gerais, Brazil) in an automated biochemical analyzer.

Tissue preparation for oxidative stress parameters

The homogenization was performed using a ground glass-type Potter–Elvehjem homogenizer. Fresh tissue was homogenized in 1.5% KCl. The homogenates were centrifuged at 800 × g for 10 min at 4°C, the pellet was discarded, and the supernatants were kept at −70°C until assays.

Oxidative stress measurements

Thiobarbituric acid-reactive substances (TBARS) measurement: The Thiobarbituric Acid-Reactive Substances (TBARS) assay was used to determine lipid peroxidation and was measured according to the method described by Ohkawa et al [23]. Briefly, 50μL of 8.1% Sodium Dodecyl Sulfate (SDS), 375μL of 20% acetic acid (pH 3.5) and 375μL of 0.8% Thiobarbituric Acid (TBA) were added to 200μL of homogenate and the mixture was incubated in a boiling water bath for 60 min. After cooling, the mixture was centrifuged (1000× g, 10 min). The supernatant was removed, and the absorbance was read at 535nm in a spectrophotometer. Commercially available malondialdehyde was used as a standard. Results were expressed as mmol/mg protein.

Carbonyl Assay: The carbonyl assay was used to determine oxidative damage to proteins. Homogenates were incubated with 2,4 dinitrophenylhydrazine (DNPH, 10mM) in 2.5 M HCl for 1 h at room temperature, in the dark. Samples were mixed every 15 min. Next, 20% (w/v) trichloroacetic acid (TCA) was added to the tubes, which were then left in ice for 10 min and centrifuged for 5 min at 1000 × g, to collect the protein precipitates. Another wash was performed with 10% TCA. The pellet was washed 3 times with ethanol/chloroform (1:1) (v/v). The final precipitates were dissolved in 6 M guanidine hydrochloride, and the solutions were used to stand for 10 min at 37°C and then read at 360 nm [24]. The results were expressed as mmol/mg protein.

Sulphhydryl 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 determined spectrophotometrically at 412 nm [25]. Briefly, 0.1mM DTNB was added to 120μL of the samples. This was followed by 30-min incubation at room temperature in a dark room. Absorbance was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as mmol/mg protein.

Determination of antioxidant enzyme activities

Superoxide Dismutase (SOD) activity, expressed as USOD/mg protein, was based on the decrease in the rate of autocatalytic adrenochrome formation at 480 nm [26]. Catalase (CAT) activity was determined by following the decrease in Hydrogen Peroxide (H2O2) absorbance at 240 nm and expressed as UCAT/mg protein [27].

Protein Determination

Protein concentrations were determined by the method of Lowry et al. [28] using bovine serum albumin as the standard.

Statistical analysis

The grape juices composition was analyzed by student t-test. All other data were analyzed by three-way Analysis Of Variance

Table 1: White grape juices composition.

<table>
<thead>
<tr>
<th></th>
<th>Density</th>
<th>Total carbohydrates</th>
<th>Total phenolic content</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGJ</td>
<td>1.06 ± 0.001</td>
<td>16.9 ± 0.1</td>
<td>7.64 ± 0.03</td>
</tr>
<tr>
<td>OGJ</td>
<td>1.04 ± 0.001</td>
<td>12.4 ± 0.01*</td>
<td>35.26 ± 0.01*</td>
</tr>
</tbody>
</table>

Total carbohydrates (g/100g); Total phenolic content (mg catechin/mL); * p<0.05, student t-test. CGJ= conventional grape juice; OGJ= organic grape juice.

Table 2: Effect of white grape juices and carbon tetrachloride (CCl₄) on biochemical parameters of Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CCl₄</th>
<th>OGJ</th>
<th>OGJ+CCl₄</th>
<th>CGJ</th>
<th>CGJ+CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>97.60 ± 4.71</td>
<td>176.00 ± 17.91*</td>
<td>93.33 ± 6.05</td>
<td>183.75 ± 23.09*</td>
<td>110.86 ± 2.42</td>
<td>169.71 ± 11.82*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>107.60 ± 14.11</td>
<td>125.40 ± 15.68</td>
<td>128.67 ± 15.45</td>
<td>120.00 ± 14.55</td>
<td>120.29 ± 7.23</td>
<td>117.75 ± 10.12</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>57.40 ± 3.34</td>
<td>40.00 ± 2.86*</td>
<td>57.66 ± 3.37</td>
<td>53.50 ± 3.63</td>
<td>61.50 ± 3.27</td>
<td>52.14 ± 3.35</td>
</tr>
<tr>
<td>HDL</td>
<td>36.60 ± 2.05</td>
<td>21.40 ± 2.27*</td>
<td>32.66 ± 1.58</td>
<td>31.12 ± 1.80</td>
<td>33.14 ± 1.12</td>
<td>32.87 ± 1.76</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.50 ± 0.04</td>
<td>0.80 ± 0.12</td>
<td>0.53 ± 0.06</td>
<td>0.85 ± 0.17</td>
<td>0.57 ± 0.02</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>Urea</td>
<td>57.40 ± 2.52</td>
<td>83.60 ± 5.88</td>
<td>50.50 ± 2.09</td>
<td>87.25 ± 9.40</td>
<td>47.00 ± 2.87</td>
<td>75.12 ± 12.19</td>
</tr>
<tr>
<td>ALT</td>
<td>50.00 ± 7.90</td>
<td>204.20 ± 21.87*</td>
<td>46.66 ± 5.11</td>
<td>137.00 ± 9.68</td>
<td>52.42 ± 6.96</td>
<td>142.75 ± 10.13</td>
</tr>
<tr>
<td>AST</td>
<td>208.60 ± 9.46</td>
<td>284.20 ± 19.44*</td>
<td>225.67 ± 12.01</td>
<td>235.13 ± 20.01</td>
<td>232.20 ± 7.93</td>
<td>211.14 ± 30.80</td>
</tr>
<tr>
<td>GGT</td>
<td>24.20 ± 6.41</td>
<td>159.80 ± 11.57*</td>
<td>46.66 ± 5.11</td>
<td>167.00 ± 39.67*</td>
<td>52.42 ± 6.96</td>
<td>182.75 ± 30.10*</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. Statistically significant differences were determined by three-way ANOVA followed by the Tukey test: *p < 0.05, different from control. CCl₄ = Carbon tetrachloride; OGJ= organic grape juice; CGJ= conventional grape juice. N=6-8/group.
Regarding to renal profile, both CCl4 and white grape juices were not able to significantly change urea and creatinine.

**Effect of grape juice and CCl4 treatment on oxidative stress parameters**

TBARS and carbonyl assays were used to determine the levels of lipid and protein damage in different brain areas of rats (Figure 1 and 2 respectively). It can be observed that CCl4 enhanced lipid peroxidation in cerebral cortex and hippocampus of rats (Figure 1) and also that CCl4 elicited oxidation of proteins in all brain areas studied (Figure 2). Figure 1 also demonstrates that OGJ and CGJ prevented the increase of TBARS provoked by CCl4 in hippocampus (Figure 1B) while only the OGJ prevented the damage caused by CCl4 in cerebral cortex (Figure 1A). Moreover, Figure 2 displays that OGJ and CGJ prevented the enhance of carbonyl caused by CCl4 in all brain areas studied (Figure 2A, B and C).

Figure 3 demonstrates the effect of CCl4 on the non-enzymatic antioxidants defenses (sulphydryl). CCl4 significantly decreased total sulphydryl levels in the hippocampus. In addition, only OGJ was able to prevent this inhibition (Figure 3B). It can also be observed in figure 3C that OGJ in the presence or absence of CCl4 increased sulphydryl content in the cerebellum of rats.
grapes, wine and purple grape juice consumption, such as reduction of cardiovascular disease risk, type 2 diabetes, and certain types of cancers [31,32]. These effects are due to their beneficial properties in preventing platelet aggregation, LDL and DNA oxidation, coronary diseases and atherosclerosis [16,33]. Moreover, the neuroprotective potential of grapes polyphenols are also described in neurological diseases such as Alzheimer’s and Parkinson’s diseases [34,35]. Considering that only few studies demonstrated the beneficial properties of white grape juices [12,20,36] the aim of this study was to observe the antioxidant and neuroprotective effect of white grape juices from Vitis labrusca (Niagara variety) on the damage provoked by CCl₄ in the brain and serum of rats.

Nowadays Brazil is producing two distinct classes of grape juices, the conventional cultivation, made from grapes that have been treated with pesticides, such as herbicides and fungicides, and also the organic cultivation, which is produced from grapes that have not received any kind of chemical or genetic manipulation [13, 37]. In the present study we observed that the OGJ had a higher content of total phenolic compounds compared to CGJ and that CGJ had a higher concentration of total carbohydrates compared to OGJ. These data are in line with previous studies form our group that also demonstrated a higher concentration of total polyphenols in OGJ and higher levels of total carbohydrates in CGJ in grape juices [12,13,38]. It is well described that the organic cultivation has a higher level of polyphenols because when pesticides are not used the plants become more susceptible to pathogens action which stimulates the production of high levels of phenolic compounds as a self-defense [8,39].

Our present study showed that CCl₄, changed the levels of glucose, total cholesterol, HDL, ALT, AST and GGT. This is in accordance with a previous study that observed hepatocellular lesion related to increases in the levels of AST and ALT [33]. Another study describes the effect of an acute treatment with different doses of an organoselenium in rats. This toxic compound decreased the levels of total cholesterol and increased the levels of AST [40]. Patel et al. [41] have found similar results evaluating healthy male grape garden pesticide sprayers exposed to pesticides within 3-10 years. There was a significant increase in AST and ALT levels on the male exposed to pesticides. Moreover, Tan et al. [42] showed that the high-fat diet increased the levels of the liver enzymes.

We also verified here that OGJ and CGJ partially prevented the biochemical changes provoked by CCl₄. Shin and Moon [43] observed that grape skin or grape seeds prevented the increased of AST and ALT caused by dimethylnitrosamine. Moreover, grape seed extract prevented the enhanced of ALT in patients with nonalcoholic fatty liver disease [44]. On the other hand, grapevine leaf extract from Bordo variety did not change the levels of total cholesterol but reduced LDL, VLDL and AST levels in diabetic rats [45].

Further biochemical analyzes also examined the effects of CCl₄, OGJ and CGJ in cellular redox status. Therefore, oxidative stress is considered a disturbance in the balance between the production of reactive species and antioxidant defenses, which can damage DNA, proteins and lipids, leading to apoptosis or necrosis in living cells [46,47]. Biological oxidative stress is controlled by the endogenous antioxidants, including the scavenger antioxidant enzymes SOD, CAT, and Glutathione Peroxidase (GPX) and exogenous dietary antioxidants, including vitamin E, C, carotenoids, and flavonoids [5].

We also investigated the enzymatic antioxidant defenses by measuring CAT and SOD activities in different brain areas of rats (Figures 4 and 5, respectively). Figure 4 shows that CAT activity was increased in cerebral cortex and reduced in hippocampus of rats by CCl₄ (Figures 4A and B). OGJ and CGJ prevented the enhance of CAT in cerebral cortex of rats (Figure 4A). Moreover, OGJ and CGJ in the presence of absence of CCl₄ reduced CAT activity (Figure 4B).

Figure 5 displays that SOD activity was reduced by CCl₄ in all tissues studied. It also can be noticed that both OGJ and CGJ were able to prevent this reduction in cerebellum (Figure 5C). However, only OGJ prevent the reduction of SOD in the hippocampus (Figure 5B) and none of the juices prevent the inhibition in cerebral cortex (Figure 5A). Moreover, GGJ per se also reduced SOD activity in the hippocampus (Figure 5B).

**Discussion**

The harmful effects caused by oxidative stress could be retarded or even reversed by increasing antioxidant levels, particularly phytochemicals such as polyphenols. The consumption of nutrients containing phenolic compounds has been reported due to the benefits they produce on human health [9,15,17]. In this context, grapes are a rich source of these compounds [12,29,30] and previous studies have confirmed a wide variety of health-promoting effects of...
Here we demonstrated that CCl4 increased TBARS levels in cerebral cortex and hippocampus of the rats and that CCl4 also enhanced carbonyl levels in all brain structures studied. On the other hand, OGJ and CGJ were able to prevent TBARS enhance in hippocampus while OGJ prevented the damage in cerebral cortex. However, OGJ and CGJ prevented carbonyl increase in all tissues. These results are in line with similar studies from our group that demonstrated that high-fat diet increased lipid peroxidation in cerebral cortex and hippocampus of rats and purple OGJ and CGJ prevented the enhanced in carbonyl levels in both tissues whereas only OGJ prevented the increase of TBARS in cerebral cortex of rats [13]. Moreover, Rodrigues et al. [19] observed that a convulsant drug, pentylenetetrazole (PTZ), induced an increase in lipid peroxidation (TBARS), and protein damage (carbonyl) in the cerebral cortex, cerebellum and hippocampus of the rats and that purple OGJ and CGJ prevented these changes. Dani et al [17] also showed that purple grape juices prevent the damage caused by CCl4 in substantia nigra of rats. A recent study demonstrated that the acute consumption of organic and conventional purple grape juices promoted a significant decrease of lipid peroxides in serum and TBARS levels in plasma in healthy individuals. Moreover, organic grape juice ingestion promoted a higher protection against serum lipid peroxidation than that of conventional grape juice [48]. These results are in accordance with our present data and with the higher content of bioactive polyphenol compounds in the organic grape juice, which could play a beneficial role against oxidative stress.

In this study we also observed that CCl4 significantly decreased the non-enzymatic antioxidant defenses in the hippocampus and that only OGJ was able to prevent this inhibition. The results of Rodrigues et al. [19] corroborates with our present results because purple grape juices were able to prevent the reduction of sulphydryl in cerebral cortex, hippocampus and cerebellum of rats after the treatment with PTZ. High-fat diet treatment reduced the sulphydryl content in cerebellum and cerebral cortex of rats and purple grape juices prevented this reduction [13]. Furthermore, the in vitro treatment with white OGJ and CGJ prevented the reduction in sulphydryl in cerebral cortex of rats after the treatment with sodium azide [20]. Interestingly we verified in the present study that OGJ increased sulphydryl content in the cerebellum in the presence or absence of CCl4. The same enhance of sulphydryl content was also verified in the treatment with purple OGJ in the study of Rodrigues et al. [19] and in the study of Ongaratti et al [20].

We also observed here that CAT activity was increased by CCl4, in cerebral cortex and reduced in the hippocampus. SOD activity was reduced by CCl4 in all tissues studied. The enhancement of CAT in the cerebral cortex was prevented by both juices but the inhibition in the hippocampus was not prevented by any of them. SOD reduction was prevented by OGJ in all tissues and CGJ just prevented this alteration in cerebellum. These results are in line with Cardozo et al. [13] that observed that a high-fat diet reduced CAT and SOD activities in different brain areas of rats and that purple OGJ and CGJ were able to prevent CAT and SOD inhibition. Moreover, PTZ also changed the activity of CAT and SOD and purple grape juices prevented these alterations [19]. Ongaratti et al [20] verified that sodium azide inhibited the activity of CAT and SOD and that white OGJ and CGJ were able to prevent the inhibition of CAT but not SOD.

Furthermore, the oral administration of a polyphenolic white grape juice extract (20 and 40 mg/kg/day) exert neuroprotective effects in an experimental mice model of autoimmune encephalomyelitis, the most commonly used model for multiple sclerosis in vivo. It reduced the clinical signs and main markers of inflammation, oxidative stress and apoptosis (TNF-α, iNOS, Nitrotyrosine, PARP, Foxp3, Bcl-2, Caspase 3 and DNA fragmentation) [49]. In this line your group recently described that OGJ and CGJ did not change the feeding behavior of the rats however both white juices were able to restore the activity of creatine kinase and pyruvate kinase (enzymes with a central role in brain energy metabolism) in different brain structures of rats [36].

We postulated that the different effects observed in the different brain areas could be provoked because the antioxidant systems are not evenly distributed across the brain tissues and this heterogeneity might implies in differential sensitivity of regions in response to chemical exposures associated with oxidative stress [50-52].

Taken together, CCl4 induced lipid peroxidation, protein damage, significantly compromised the non-enzymatic and the enzymatic antioxidant defenses and increased the levels of reactive species in the brain of rats. As a result, there was an unbalance between pro-oxidants and antioxidants, a situation defined as oxidative stress [1,3]. White grape juices were capable to prevent or to ameliorate this condition, being OGJ, which is richer in polyphenol content, more effective in this protection. Moreover, considering that it is well described in the literature, the association between oxidative stress and diseases that affect the central nervous system, we could speculate that regular intake of grape products could be considered as an adjuvant in the therapy of patients with these diseases.

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