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Assessment of changes in energy metabolism parameters provoked by carbon tetrachloride in Wistar rats and the protective effect of white grape juice

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ABSTRACT

The objective of this study was to evaluate the effect of organic and conventional grape juices consumption on the behavior of rats and their neuroprotective effect on the activity of brain energy metabolism enzymes in different brain areas of adult rats on the experimental model of hepatic encephalopathy. Male Wistar rats (90-days-old) were treated once a day with conventional or organic white grape juice by gavage for 14 days (7 μL/g). On the 15th day the rats received carbon tetrachloride (CCl_4) in a single dose of 3.0 mL/kg. Cerebral cortex, hippocampus and cerebellum were dissected to measure the activity of creatine kinase (CK) and pyruvate kinase (PK). No changes in feeding behavior were observed after the treatment with the grapes juices. However, there was an increase in grooming behavior in the open field test provoked by both juices. CCl_4 inhibited CK activity in cerebral cortex and hippocampus of the rats and CCl_4 also reduced PK activity in all brain structures studied. Furthermore, both white grape juices prevented the decrease in the activity of CK and PK. Therefore, we can suggest that organic and conventional white grape juices could restore the activity of enzymes with a central role in brain energy metabolism.

1. Introduction

Hepatic encephalopathy is a neuropsychiatric syndrome which is frequently in patients with liver disease or portal hypertension [1]. One of the experimental models used to study this disease uses carbon tetrachloride (CCl_4), which is a potent hepatotoxic drug [2,3]. When CCl_4 is metabolized by hepatocytes, there is a stimulation of reactive species and free radicals formation that can lead to tissue damage [2–4]. However, the mechanisms underlying hepatic encephalopathy are still not completely understood, nevertheless it has been suggested that ammonia and toxins are able to cross into the systemic circulation by the portal blood flow [5]. Then, ammonia penetrates into the brain tissue and triggers alterations in energy

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metabolism, oxidative stress, activation of peripheral benzodiazepine receptors and neurotransmitter systems dysfunction [6].

The brain has a high rate of oxidative metabolism, consuming approximately 20% of the cardiac output [7]. Therefore, coupling of separated intracellular adenosine triphosphate (ATP) producing and ATP-consuming processes is fundamental to the function of living organisms. On the other hand, the spatial arrangements of energy are insufficient for all cellular energetic requirements [8], being necessary an enzymatic network, catalyzed by creatine kinase (CK) and glycolytic enzymes, in especial pyruvate kinase (PK), to support high-energy phosphoryl transfer between ATP-generating and ATP-consuming processes [9–11]. This dynamic metabolic signaling maintains the balance between cellular ATP consumption and production, and the energetic homeostasis for preserving cell survival [9,12]. However, this homeostasis can be interrupted by an increased production of free radicals and reactive species [13].

In this context, phenolic compounds present in foods and beverages produce beneficial physiological effects on human body functioning as antioxidants agents [14–16]. There are various types of grape juices in the world market. In Brazil grape juices are mainly produced with grapes from *Vitis labrusca* species. The Niagara variety from *V. labrusca* is considered the American grape mainly used for production of table wines. This variety is widely consumed *in natura* as also in juice because of its characteristic aroma and flavor [17]. Rio Grande do Sul, the largest grape producer state in Brazil, in 2014 has marketed a total of 2487.714 L of white juices [18].

Nowadays, there are two classes of grape juices, conventional and organic. The conventional grape juice (CGJ) is produced from grapes grown in the presence of insecticides, herbicides and fungicides and the organic grape juice (OGJ) is produced from grapes that grown free of pesticides and/or genetic engineering [19–21]. The organic purple grape juices have higher concentrations of resveratrol and polyphenol content compared to with the grapes that grown with conventional production methods [20]. The daily consumption of purple grape juice can promote a reduction in levels of circulating lipids, blood pressure and oxidative damage to DNA, and also an inhibition of platelet aggregation and the prevention of some diseases [19,20,22–24].

Considering that the pathophysiological changes of neurodegenerative diseases are associated with disorders of energy metabolism [25–27], that it is well described in the literature the beneficial effects of purple grape juice [19,22,23,28,29], and until the moment there are no studies with white grape juice in an animal model *in vivo*. The objective of this study was to evaluate the neuroprotective effect of CGJ and OGJ from *V. labrusca* (Niagara variety) on the activity of CK and PK in the cerebral cortex, hippocampus and cerebellum of rats and also to verify the effect of the consumption of white grape juices on feeding behavior and on the open field test in Wistar rats.

2. Material and methods

2.1. Chemicals

Carbon tetrachloride (CCl_4), tris, NADH, phosphocreatine and ADP were purchased from Sigma Chemical Co. (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 *V. labrusca* 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 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 by extraction, with a subsequent pressing in order to separate the pulp, then submitted to pasteurization (at 85 °C), and immediately followed by bottling at 80 °C.

2.3. Grape juices chemical evaluation and phenolic compound content

Total acidity, volatile acidity, total carbohydrates, density and pH were determined according to AOAC International official methodologies [30]. Total phenolic content was measured using Singleton and Rossi's modification of Folin–Ciocalteau's colorimetric method.

2.4. Animals

Forty adult male Wistar rats (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.

2.5. Treatment

The animals were randomly divided into three experimental groups. Group 1 received water; Group 2 received CGJ (7 μL/g of body weight); Group 3 received OGJ (7 μL/g of body weight). All animals were orally administered (gavage) with a single dose of CGJ, OGJ or water during 14 days. On the 15th day half of the animals in each group received a single intraperitoneal injection of CCl_4 at the dose of 3.0 mL/kg and the other half of the animals received intraperitoneally only vehicle (mineral oil). After 4 h the animals were euthanized by decapitation and the trunk

blood and the cerebral cortex, the hippocampus and the cerebellum were dissected and kept chilled until homogenization.

2.6. Body composition

Animal body weight was assessed daily on electronic balance (Crystal 200, Gibertini, Italy). The data were expressed as weight in grams per animal.

2.7. Evaluation of food and drink consumption

Food and water intake was controlled daily. The consumption of food and water was measured by the difference between the initial and final weight in a period of 24 h, the results were expressed in grams per day.

2.8. Open field test

The effect of the grape juices consumption was evaluated on the 14th day. The animals were individually placed in a 20 cm × 30 cm × 50 cm wooden box divided in 12 squares by lines painted on its floor. Behavior analyses were performed in a quiet room during 5 min and the number of the squares crossed with the four paws, number of rearing responses, number of grooming and number of fecal boli were recorded by an observer not aware of the subject condition [31]. Crossing and rearing are considered exploratory behavior, while grooming and fecal boli are considered expressions of emotionality [32]. The number of squares crossed is indicative of motor activity and its reduction along the session, a measure of habituation [31]. Higher levels of anxiety should lead to a decrease in the number of squares visited in center. The reduction in the number of rearing responses along the session is also considered as a measure of habituation.

2.9. Biochemical parameters

The trunk blood was collected in tubes without any anticoagulant. Serum was obtained by centrifugation at 1000 × *g* for 10 min (hemolyzed serum was discarded). Hepatic function was analyzed using alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) activities as markers of toxicity. All assays were carried out using commercial kits in an automated biochemical analyzer.

2.10. Tissue preparation for CK and PK activity

The cerebral cortex, the hippocampus and the cerebellum were kept approximately at 0 °C and then homogenized with a sucrose solution (0.32 mol/L Tris 65 mM, EDTA 10 mM), pH 7.4. The tissues were washed in 0.89% saline solution, minced finely and homogenized (1:10, w/v with a Potter-Elvehjem glass homogenizer). At least two homogenizations of 30 s duration were performed at approximately 1000 rpm with an electrically driven Teflon pestle.

2.10.1. CK activity

The homogenates containing approximately 1 μg of protein were pre-incubated at 37 °C for 5 min in a mixture containing the following final concentrations: 7 mM phosphocreatine, 9 mM $MgSO_4$, 65 mM Tris–HCl buffer, pH 7.5 in a final volume of 0.1 mL. Incubation was started by the addition of 3.2 mM adenosine diphosphate (ADP). The reaction was stopped after 10 min by the addition of 10 mmol *p*-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of [33]. The color was developed by the addition of 0.1 mL 2% α-naphthol and 0.1 mL of 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. All assays were performed in triplicate and the mean was used for the calculations. No substance added to the assay interfered with the color development and reading. Results were obtained as μmol of creatine formed per min per mg of protein.

2.10.2. PK activity

The enzymatic activity of PK was performed as described by [34]. A portion of the homogenate was centrifuged at 10,000 × *g*, the pellet was discarded and the supernatant containing cytosol and other cellular components as endoplasmic reticulum was collected for determination of PK activity. The reaction solution containing 0.1 M Tris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 unit of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100 and 10 mL of the mitochondria-free supernatant in a final volume of 0.5 mL. After 30 min of pre-incubation at 37 °C, the reaction was initiated by the addition of 1.0 mM phosphoenolpyruvate (PEP). NADH oxidation was evaluated spectrophotometrically by 2 min at 340 nm. The results were expressed in μmol of pyruvate formed/min/mg protein.

2.11. Protein determination

Protein concentrations were determined by the method of [35] using bovine serum albumin as standard.

2.12. Statistical analysis

The grape juices composition and the biochemical parameters were analyzed by Student's *t*-test. Changes on body weight gain and food and drink intake were analyzed by one-way-ANOVA for repeated measures. All other data were analyzed by three-way analysis of variance (ANOVA) followed by Tukey test to determine differences between groups. 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

3.1. Grape juices composition

Table 1 shows the composition of CGJ and OGJ. We observed that both grape juices showed the same total

Table 1
White grape juices composition.

	Conventional grape juice	Organic grape juice
Total acidity	0.65 ± 0.001	0.64 ± 0.001
Volatile acidity	0.04 ± 0.01	0.04 ± 0.01
Total carbohydrates	16.9 ± 0.1	12.4 ± 0.01*
Density	1.06 ± 0.001	1.04 ± 0.001
pH	3.58 ± 0.01	3.89 ± 0.01
Total phenolic content	7.64 ± 0.03	35.26 ± 0.01*

Total acidity (g% tartaric acid), volatile acidity (g/100 g), total carbohydrates (g/100 g); total phenolic content (mg catechin/mL); data are mean ± standard deviation.
* $P < 0.05$, Student's *t*-test.

acidity, volatile acidity, density and pH. However, total carbohydrates concentration was higher in the CGJ as compared to the OGJ and the total phenolic content of the OGJ was significantly higher than the CGJ.

3.2. Effect of grape juices treatment on body weight gain and food and drink intake

No changes were observed in the pattern of food (Fig. 1A) and water (Fig. 1B) intake by the animals during the period of treatment. We also did not notice any modifications in body weight gain in the animals treated with CGJ or OGJ during the experiment (Fig. 1C).

3.3. Effect of grape juices treatment on the behavior in the open field test

Treatment with the CGJ or OGJ increased the number of grooming in the rats (Table 2). On the other hand, statistical analysis of the latency to locomotion, total, peripheral and central ambulation, number of rearing responses and fecal boli did not show any differences between the groups (Table 2).

3.4. Effect of CCl_4 treatment on the biochemical parameters

In order to evaluate if the experimental model is mimicking the hepatic encephalopathy in the rats we evaluated the markers of hepatic toxicity ALT, AST and GGT. It was observed that CCl_4 enhanced ALT, AST and GGT activities compared to the group that did not receive CCl_4 (Table 3).

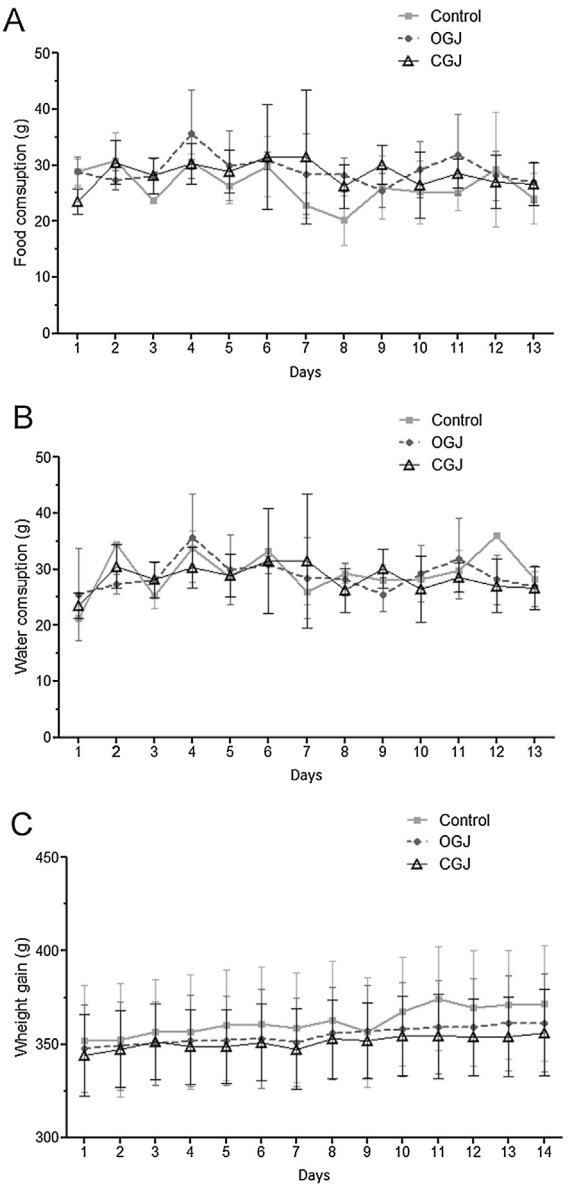

Fig. 1. Effect of treatment with organic and conventional white grape juices on food consumption (A), water consumption (B), and weight gain (C) in rats. Data are expressed as mean ± standard deviation and statistical analysis was analyzed by one-way-ANOVA for repeated measures. OGJ, organic grape juice; CGJ, conventional grape juice. $N = 10$ animals/group.

Table 2
Effect of grape juices treatment on the behavior in the open field test.

	Control	Organic grape juice	Conventional grape juice
Latency	3.40 ± 1.83	7.20 ± 3.43	6.64 ± 2.40
Total ambulation	75.60 ± 11.21	68.20 ± 27.42	65.42 ± 19.07
Peripheral ambulation	64.10 ± 12.32	56.60 ± 27.24	61.50 ± 17.19
Central ambulation	11.50 ± 5.67	3.60 ± 2.24	3.93 ± 3.05
Rearing	25.60 ± 7.74	26.47 ± 5.13	20.00 ± 7.54
Grooming	4.20 ± 1.30	18.07 ± 6.87*	14.71 ± 4.26*
Fecal boli	2.00 ± 0.38	1.60 ± 0.72	1.50 ± 0.76

Latency (seconds); total ambulation, peripheral ambulation, central ambulation, rearing, grooming and fecal boli (frequency). Statistically significant differences were determined using ANOVA followed by Tukey test; data are mean ± standard deviation.
* $P < 0.05$, from control.

Table 3
Effect of carbon tetrachloride (CCl_4) on the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) in serum of rats.

	Control	CCl_4
ALT (U/L)	50.00 ± 7.90	204.20 ± 21.87*
AST (U/L)	208.60 ± 9.46	299.78 ± 39.44*
GGT (U/L)	20.20 ± 6.41	80.59 ± 11.57*

Data are mean ± standard deviation.
* $P < 0.05$, Student's *t*-test.

3.5. *Effect of CCl_4 and grape juices treatment on the activity of CK and PK*

The effect of the CGJ or OGJ on the activity of CK and PK in the cerebral cortex, hippocampus, and cerebellum of rats treated with CCl_4 was observed (Figs. 2 and 3, respectively). CCl_4 inhibited the activity of CK in the cerebral cortex (Fig. 2A) and hippocampus (Fig. 2B), without changing the activity of this enzyme in the cerebellum (Fig. 2C) of the rats. Moreover, CGJ and OGJ were able to prevent the decrease in CK activity in cerebral cortex and hippocampus (Fig. 2).

It was also verified that the CCl_4 inhibited the activity of PK in all brain structures studied (Fig. 3A–C). Furthermore, both CGJ and OGJ were able to prevent this decrease in the cerebral cortex, hippocampus, and cerebellum of the animals (Fig. 3).

4. Discussion

In the present study, we determined the composition of CGJ and OGJ and identified significant differences in the amount of total carbohydrates, being CGJ richer in sugars as compared to OGJ. On the other hand, OGJ had a higher amount of total phenolic compounds compared to CGJ. These results corroborate with a study from [20] which evaluated the amount of polyphenols in purple and white CGJ and OGJ of *V. labrusca* and found that the highest concentration of total phenolic compounds in juices from organic grapes. Also [19] observed that purple CGJ had more total carbohydrates compared to the OGJ.

Our results showed no changes in the feeding behavior of food and water consumption in the animals treated with CGJ or OGJ. We also observed no alteration in the weight of these animals during the experimental period. This is in line with a controlled clinical study of [36] that showed that regular consumption of Concord grape juice for 12 weeks was not able to promote weight gain in adult volunteers. Other studies with children and adolescents who consumed 100% fruit juices demonstrated that volunteers began to consume a more nutritious diet and, thus, had no increase in weight [37,38]. Therefore, the daily intake of grape juice associated with a balanced diet does not cause significant effects on body weight gain [39,40].

Here we also demonstrated that the treatment with both CGJ and OGJ increased the number of grooming performed by the animals in the open field test. Since the grooming is spontaneous in some species [41,42], the increase execution of this parameter in the animals may indicate two opposite situations, as behavioral reactions caused by changes that stimulate the increase or decrease in tension [43,44]. Our results could corroborate with [45] that found an increase in grooming after the administration of clonazepam in female rats, suggesting an anxiolytic effect caused by the drug. Moreover, a in recent study [46] showed that the daily intake of organic purple grape juice for 7 days showed an anxiolytic effect in animals that were exposed to x-irradiation due to a decrease in the frequency of access to the center and corners in open field test and the increase in the total stay in corners in the open field test.

The serum aminotransferases are sensitive enzymes that indicate damage to hepatocytes [47]. In our study we observed that CCl_4 increased the activity of ALT, AST and GGT in the serum of the animals when compared to the control group, indicating hepatic failure in our experimental model. Our results corroborate with [48] that verified an increase of ALT and AST in the serum of the rats treated with CCl_4, suggesting an increase in permeability damage and/or necrosis of hepatocytes. Thus, we can assure that the technique used in this study was able to induce hepatic encephalopathy. In this disease the cerebral intoxication occurs due to liver metabolism of non-nitrogenous substances, which increase mainly the levels of ammonia in the brain which can lead to brain edema by increasing the volume of astrocytes [49]. The stimulation of the production of free radicals and reactive species is caused by the metabolism of CCl_4 by the P450 cytochrome [4]. This mechanism causes tissue fibrosis and the death of the hepatocytes [50].

In this context, CK, a key enzyme for the regulation of cerebral metabolism, that is composed of a group of five isoenzymes that act primarily in tissues that require a high consumption of energy, such as brain (CK-BB), cardiac muscle (CK-MB), skeletal muscle (CK-MM) and the mitochondrial isoforms that act in intermembranes spaces (CK-Mia) and striated muscle (CK-Mib) [9,51]. Moreover [27], suggested that alterations in CK activity can cause neurodegenerative diseases which could lead to neural loss and cerebral dysfunction.

In the present study we noticed an inhibition of CK activity in the cerebral cortex and hippocampus of rats that received CCl_4. However, it was also observed that the rats that received a pre-treatment with CGJ or OGJ and afterwards received CCl_4 restored CK activity in the cerebral cortex and hippocampus, suggesting that both grape juices could prevent the reduction of CK activity caused by CCl_4. In line with this result other studies demonstrate that oxidative stress could be a participant factor in several diseases, in particular neurodegenerative diseases that are characterized by selective neuronal death [52,53]. In this context, some studies suggest that an inhibition of CK could be related to oxidative stress, because the CK may be the first target of reactive oxygen species [54–56]. Moreover, reduction to CK function could cause neurodegeneration that may lead to neuronal loss in the brain, since energy is required for the development and regulation of brain functions [27].

It is well established that CK decreases its activity in Alzheimer's disease and other neurodegenerative diseases [57]. A study by [58] in which patients with Alzheimer's disease showed that in the early stages of the disease the levels

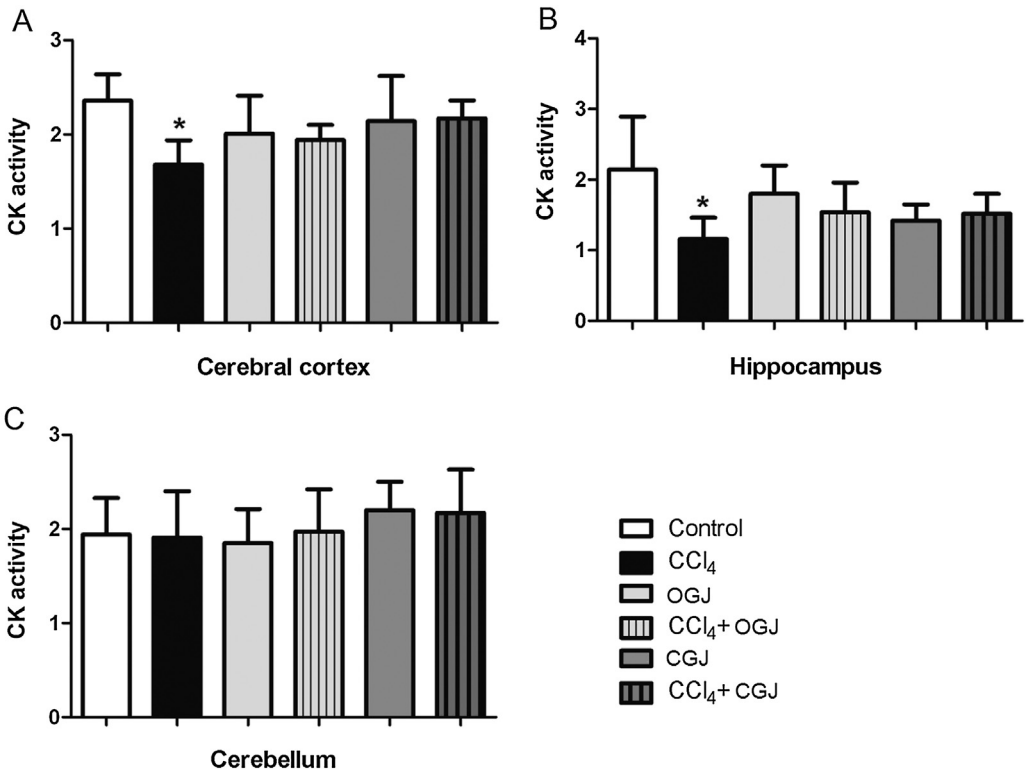

Fig. 2. Effect of treatment with organic and conventional white grape juices on the activity of creatine kinase (CK) activity in the cerebral cortex (A), hippocampus (B) and cerebellum (C) of rats treated with carbon tetrachloride (CCl_4). Data are expressed as mean ± standard deviation and statistical analysis was analyzed by three-way ANOVA followed by post-Tukey test. $^*P < 0.05$ different from all groups. OGJ, organic grape juice; CGJ, conventional grape juice. $N = 6–8$ animals/group.

of phosphocreatine are reduced and in later stages of the disease patients have a reduction in oxidative metabolism compared to healthy people, indicating that brain energy changes in patients with Alzheimer's disease. Zhang et al. [59] using brain samples from mice and patients with Huntington's disease showed a significant decrease in CK activity, confirming the hypothesis that energy metabolism is impaired in this disease. Quincozes-Santos et al. [60] showed that resveratrol was able to prevent the reduction of the activity of CK induced by H_2O_2 in glial cells cultures. Other study by [61] showed the protective effect of quercetin on the activity of CK in the hippocampus of adult rats exposed to polychlorinated biphenyls (PCBs), where quercetin supplementation was able to restore the activity of CK.

Although the metabolic activity of the brain is intense, the cerebral energy storage is extremely small, and therefore there is requirement of a constant amount of glucose, as it is the main energy substrate for the brain [62]. In this context, glycolysis and oxidative phosphorylation are the main biochemical pathways of energy production for the mammalian brain [63]. In most cases the primary fuel is ATP that when hydrolyzed releases energy [64]. In situations of reduced energy metabolism, brain tissue is susceptible to damage because it is dependent on a continuous supply of energy [65].

PK is responsible for catalyzing the 10th reaction in the glycolytic pathway, producing ATP and pyruvate [66]. This is an allosteric enzyme activated by fructose-1,6-bisphosphate which participates in the control of rapid flow in the glycolytic pathway [67]. Therefore, PK is a thiol crucial enzyme for the glycolytic pathway and cell metabolism that is present in all tissues [68,69] and it is essential for the release of energy in the brain [70]. The inhibition of PK decreases pyruvate synthesis and could induce cell death.

In mammals, there are at least four known isozymes of PK, the isozyme L which acts in the liver, the isoenzyme which R is present in the kidney, the M1 isoenzyme that acts in the brain and muscle and, finally, the M2 isoenzyme is present in undifferentiated and proliferating tissues [71–74]. In our present work we observed a significant decrease of PK activity in the cerebral cortex, hippocampus and cerebellum of animals treated with CCl_4. However, we also noticed that the rats that received CGJ and OGJ were able to restore PK activity. It is well described in the literature that exposure to agents that promote the generation of free radicals and reactive species such as substances that react with thiol groups and amino acids have the capacity to decrease the activity of PK, probably by modifying sulfhydryl residues of the enzyme [75–78]. Moreover, inhibition of PK decreases synthesis of pyruvate, which can consequently induce cell death [79].

On the other hand, the rats that received pretreatment with CGJ and OGJ restored PK activity. Thus, some studies corroborate with our results showing that antioxidant

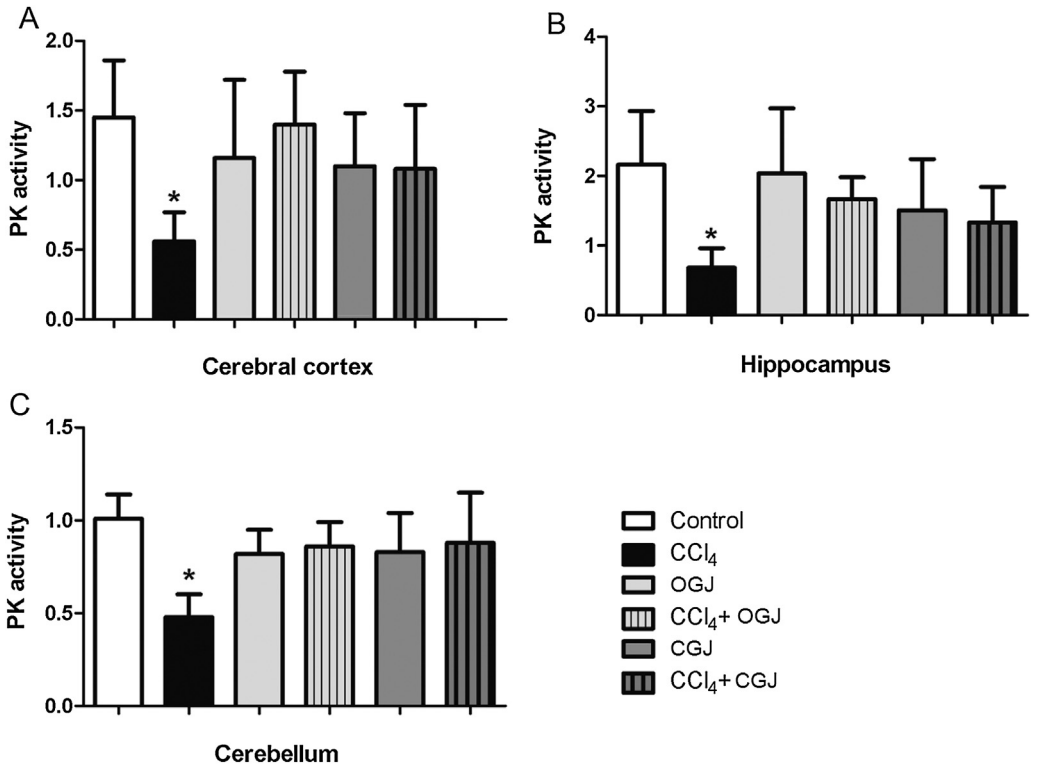

Fig. 3. Effect of treatment with organic and conventional white grape juices on the pyruvate kinase (PK) activity in the cerebral cortex (A), hippocampus (B) and cerebellum (C) of rats treated with carbon tetrachloride (CCl_4). Data are expressed as mean ± standard deviation and statistical analysis was analyzed by three-way ANOVA followed by post-Tukey test. *$P < 0.05$ different from all groups. OGJ, organic grape juice; CGJ, conventional grape juice. $N = 6–8$ animals/group.

compounds could prevent the inhibition of PK. Feksa et al. [77] verified that alanine administration both *in vivo* and *in vitro*, was capable of preventing the inhibition PK caused by the phenylalanine. Another *in vitro* study conducted by [80] showed that high doses of tyrosine, similar to the levels found in tyrosinemia type II, inhibits the activity of PK, however pre-treatment with creatine was able to prevent such inhibition, suggesting that, at least in part, changes in energy metabolism contributes to the neurological dysfunction characteristic of tyrosinemia type II.

Therefore, we propose that CGJ and OGJ are capable of preventing the inhibition of CK and PK against the damage caused by CCl_4 without change the feeding behavior and the exploratory behavior of the rats. Thus, it is feasible to propose that a diet rich in phenolic compounds could have an important beneficial role in human health, and maybe even prevent the development and progression of some neurodegenerative diseases by restoring the activity of enzymes that play a central role in brain energy metabolism.

Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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