Neurochem Res (2014) 39:1681–1690
DOI 10.1007/s11064-014-1360-9

ORIGINAL PAPER

The Protective Effect of *N*-Acetylcysteine on Oxidative Stress in the Brain Caused by the Long-Term Intake of Aspartame by Rats

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Received: 18 February 2014 / Revised: 28 May 2014 / Accepted: 10 June 2014 / Published online: 27 June 2014

Abstract Long-term intake of aspartame at the acceptable daily dose causes oxidative stress in rodent brain mainly due to the dysregulation of glutathione (GSH) homeostasis. *N*-Acetylcysteine provides the cysteine that is required for the production of GSH, being effective in treating disorders associated with oxidative stress. We investigated the effects of *N*-acetylcysteine treatment (150 mg kg^{-1}, i.p.) on oxidative stress biomarkers in rat brain after chronic aspartame administration by gavage (40 mg kg^{-1}). *N*-Acetylcysteine led to a reduction in the thiobarbituric acid reactive substances, lipid hydroperoxides, and carbonyl protein levels, which were increased due to aspartame administration. *N*-Acetylcysteine also resulted in an elevation of superoxide dismutase, glutathione peroxidase, glutathione reductase activities, as well as non-protein thiols, and total reactive antioxidant potential levels, which were decreased after aspartame exposure. However, *N*-acetylcysteine was unable to reduce serum glucose levels, which were increased as a result of aspartame administration. Furthermore, catalase and glutathione S-transferase, whose activities were reduced due to aspartame treatment, remained decreased even after *N*-acetylcysteine exposure. In conclusion, *N*-acetylcysteine treatment may exert a protective effect against the oxidative damage in the brain, which was caused by the long-term consumption of the acceptable daily dose of aspartame by rats.

Keywords *N*-Acetylcysteine · Protective · Aspartame · Brain · Oxidative damage · Glutathione

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Introduction

The artificial dipeptide sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester) is present in many products in the market, especially in unsweetened or sugar free products. It is approx. 200-fold sweeter than sugar without a bitter aftertaste [1]. Although some studies suggest that aspartame is not cytotoxic [1, 2], there is concern and research evidence suggesting possible adverse effects due to aspartame metabolic components produced during its breakdown [3].

Aspartame differs from other dietary sweeteners, since it is absorbed from the intestine and is rapidly metabolized similarly in rodents, primates, and humans by the liver to its essential constituents: aspartate, phenylalanine, and methanol [4]. The first two are known as amino acid isolates. Aspartate is a highly excitatory neurotransmitter [5]. The neutral amino acid phenylalanine is the precursor of dopamine and norepinephrine [6]. It was associated with the reported clinical and behavior effects linked to aspartame ingestion, including headaches, dizziness, and mood alterations [7]. Methanol only accounts for 10 % of the aspartame molecule [8]. Nevertheless, Trocho et al. [9]

exhibited that aspartame administration by gavage to rats (200 mg kg^{-1}) for 10 days resulted in progressive accumulation of formaldehyde, a byproduct of methanol metabolism, in the protein and nucleic acids of several sensitive tissues such as the brain, altering their cellular functions.

The debate about the safety of aspartame still continues after the Food and Drug Administration approved aspartame use in dry applications in 1981, followed by approval for use in carbonated soft drinks in 1983, and then a general sweetener in 1996 [1]. An update on the safety in use of aspartame established its acceptable daily intake by humans at 40 mg kg^{-1} [10]. Nevertheless, some recent research has shown that long-term intake of aspartame not only in acceptable (40 mg kg^{-1}) [11] but also in lower [12] and abusive doses [13–15] to rodents results in oxidative stress in the brain. It probably occurred due to the action of methanol *per se* and its metabolites (formaldehyde and formate), which are involved in the increase of reactive oxygen species (ROS) formation, altering ROS-scavenging enzymes, mainly those related to the glutathione (GSH)-dependent system [14]. Therefore, in view of the fact that aspartame consumption is essential to diabetic individuals, including children, [1] and its usage is increasing in health-conscious societies [2], compounds that could be very effective in diminishing the cumulative effects of oxidative stress, such as *N*-acetylcysteine, whose therapeutic effects are largely associated with its capability to scavenge ROS and elevate cellular GSH levels are constantly searched. Previous research showed that *N*-acetylcysteine administration [150 mg kg^{-1}, intraperitoneal (i.p.)] for 7 days exhibited a neuroprotective effect against lipid peroxidation during methanol intoxication in rats by increasing GSH levels and GSH-related enzyme activities [16].

N-Acetylcysteine has been in clinical practice for several decades. It has been used for the treatment of numerous neuropsychiatric disorders and neurological conditions, such as Alzheimer's disease. *N*-Acetylcysteine is the acetylated precursor of the amino acid L-cysteine, which sustains the synthesis of GSH [17]. GSH is the ubiquitous source of the thiol pool in the body and an important antioxidant involved in numerous physiological processes, such as the detoxification of electrophilic xenobiotics, antioxidant defense, and neurotransmitter signaling [18]. To our knowledge, there are no reports showing the effects of *N*-acetylcysteine on the brain disturbances in the antioxidant defense system and in the oxidative damage process caused by aspartame long-term exposure in rats. Hence, we investigated the effects of *N*-acetylcysteine on some oxidative stress biomarkers in the brain of rats chronically treated with aspartame.

Materials and Methods

Chemicals

All reagent-grade chemicals were obtained from Sigma (St. Louis, Missouri, USA).

Animals

Adult male Wistar rats (120–150 g) were obtained from the Central Animal Breeding Facility of the Federal University of Santa Maria, RS, Brazil. They were kept in polypropylene cages with a controlled temperature (23 ± 2 °C) and a light–dark cycle of 12 h with access to water ad libitum and to approximately 30 g daily per animal of rodent laboratory chow (Supra, São Leopoldo, RS, Brazil). The study was approved by the Ethics Committee of the Federal University of Santa Maria (#020/2012).

Experimental Protocol

Rats (n = 40) were randomly divided into four groups with ten animals. *Control group:* received only a 154 mmol l^{-1} NaCl solution by gavage for 6 weeks. Concomitantly, from the 5th week, i.p. injections of 154 mmol l^{-1} NaCl solution were administrated for 2 weeks. *NAC group:* received only a 154 mmol l^{-1} NaCl solution by gavage for 6 weeks. Concomitantly, from the 5th week, i.p. injections of *N*-acetylcysteine at a dose of 150 mg kg^{-1}, freshly prepared in a 154 mmol l^{-1} NaCl solution, whose pH was adjusted within the range of 6.8–7.2 with 6 N NaOH, were administrated for 2 weeks. *Aspartame group (ASP):* received aspartame by gavage at a dose of 40 mg kg^{-1} freshly prepared in a 154 mmol l^{-1} NaCl solution for 6 weeks. Concomitantly, from the 5th week, i.p. injections of 154 mmol l^{-1} NaCl solution were administrated for 2 weeks. *Aspartame group treated with N-acetylcysteine (ASP–NAC):* received aspartame by gavage at a dose of 40 mg kg^{-1} freshly prepared in a 154 mmol l^{-1} NaCl solution for 6 weeks. Concomitantly, from the 5th week, i.p. injections of *N*-acetylcysteine at a dose of 150 mg kg^{-1}, freshly prepared in a 154 mmol l^{-1} NaCl solution, whose pH was adjusted within the range of 6.8–7.2 with 6 N NaOH, were administrated for 2 weeks. Treatments (aspartame, *N*-acetylcysteine or 154 mmol l^{-1} NaCl solution) were administered on a daily basis (at 9:00 a.m.) and the animals' body weight was recorded weekly. At the end of 6th week and 3 h after the last treatment, the rats were weighted, anesthetized with xylazine and ketamine for the blood collection by cardiac puncture. Then, the animals were euthanized by decapitation for the removal of their brain, which was immediately weighted, cleaned and washed in an ice-cold 154 mmol l^{-1} NaCl solution.

Blood Analysis

The blood was collected in tubes and it was immediately centrifuged at 1,800*g* for 15 min at 4 °C. Serum was separated for glucose measurement by using commercial kits (Labtest, Lagoa Santa, MG, Brazil).

Tissue Homogenate Preparation

The brain was homogenized in 154 mmol l^{-1} KCl containing 1 mmol l^{-1} phenylmethylsulfonyl fluoride and centrifuged at 700*g* for 10 min at 4 °C to discard the nuclei and cell debris. The supernatant fraction obtained was frozen at −70 °C for further measurements.

Lipid Peroxidation

Lipid peroxidation, as indicated by the amount of lipid hydroperoxides (LOOH), was measured by the xylenol orange method [19]. This technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. For the assay the following reagents were added sequentially at a final concentration: 20 mol l^{-1} methanol, 100 μmol l^{-1} xylenol orange, 25 mmol l^{-1} sulfuric acid (H_2SO_4), 4 mmol l^{-1} butylated hydroxytoluene, 250 μmol l^{-1} ferrous sulfate, to a total of 0.9 ml. The sample aliquots (0.1 ml) were then added and incubated at room temperature for 30 min, and the absorbance at 560 nm was then read. The results were reported as nmol mg $protein^{-1}$ using $\varepsilon_{560} = 43$ $mmol^{-1}$ cm^{-1}. Lipid peroxidation was also estimated based on the formation of thiobarbituric reactive substances (TBARS) [20]. Briefly, the sample aliquots (0.25 ml) were added to a tube containing 0.75 ml of 1.22 mol l^{-1} trichloroacetic acid (TCA) and 0.5 ml of 46 mmol l^{-1} thiobarbituric acid. The mixture was heated for 15 min at 100 °C. After cooling, the precipitate was removed by centrifugation. The absorbance of the organic phase was measured at 535 nm, and the results were expressed as nmol mg $protein^{-1}$ using $\varepsilon_{535} = 156$ $mmol^{-1}$ cm^{-1}.

Carbonyl Protein

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound. The sample aliquots (0.2 ml) were incubated with 0.8 ml of 10 mmol l^{-1} DNPH in 2.5 mol l^{-1} HCl solution or 2.5 mol l^{-1} HCl solution (blank) for 1 h at room temperature, in the dark. Samples were vortexed every 15 min. Then, 1 ml of 1.2 mol l^{-1} TCA solution was added in the tube samples, left in ice for 10 min and centrifuged for 5 min at 1,000*g* to collect the protein precipitates. Another wash was performed with 0.8 ml of 0.61 mol l^{-1} TCA. The pellet was washed three times with 0.8 ml of ethanol:ethyl acetate (1:1). The final precipitates were dissolved in 0.4 ml of 6 mol l^{-1} guanidine hydrochloride solution, left for 10 min at 37 °C and read at 360 nm [21]. The standard curve was prepared by using different bovine serum albumin concentrations (0.5–1.5 mg ml^{-1}) and the slope was used to express the levels of carbonyl protein as nmol mg $protein^{-1}$.

Antioxidant Enzymes

Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adrenochrome generation at 480 nm. Sample aliquots (0.1 ml) were added to 2.9 ml of an assay mixture containing 1 mmol l^{-1} epinephrine and 50 mmol l^{-1} glycine (pH 10.2) at a final concentration. The enzyme activity was expressed as USOD mg $protein^{-1}$ using $\varepsilon_{480} = 4$ $mmol^{-1}$ cm^{-1}. One SOD unit was defined as the amount of enzyme required for 50 % inhibition of the adrenochrome formation [22].

Catalase (CAT) activity was evaluated by measuring the decrease in the absorption at 240 nm. Sample aliquots (0.1 ml) were added to 1.9 ml of a reaction medium consisting of 50 mmol l^{-1} phosphate buffer (pH 7.4) and 2 mmol l^{-1} H_2O_2 at a final concentration, thereby providing information to determine the pseudo-first-order reaction constant (k′) of the decrease in H_2O_2 absorption [23]. The results were reported as pmol mg $protein^{-1}$ using $\varepsilon_{240} = 40$ mol^{-1} cm^{-1}.

Glutathione peroxidase (GPx) activity was measured by using a coupled reaction with GR in the presence of GSH and NADPH, as described previously by Flohé and Gunzler [24]. The oxidized glutathione (GSSG) produced upon reduction of t-butyl hydroperoxide by GPx is reduced by GR using NADPH as a cofactor. The oxidation of NADPH to $NADP^+$ results in a decrease in the absorbance at 340 nm. Sample aliquots (0.1 ml) were added to 1.2 ml of the assay mixture containing 100 mmol l^{-1} phosphate buffer (pH 7.7), 1 mmol l^{-1} EDTA, 0.2 U ml^{-1} of GR, 2 mmol l^{-1} of GSH, 0.2 mmol l^{-1} NADPH at a final concentration. Subsequently t-butylhydroperoxide was added to a final concentration of 0.5 mmol l^{-1} and the change in the absorbance was recorded at regular intervals of a period of 3 min. The enzyme activity was expressed as μmol min^{-1} mg $protein^{-1}$ using $\varepsilon_{340} = 6.22$ $mmol^{-1}$ cm^{-1}.

Glutathione reductase (GR) is an NADPH-dependent enzyme that regenerates GSH from GSSG. Its activity was expressed as nmol min^{-1} mg $protein^{-1}$ and measured by the rate of NADPH consumption at 340 nm using $\varepsilon_{340} = 6.22$ $mmol^{-1}$ cm^{-1}. Samples aliquots (0.1 ml) were added to 0.9 ml of a reaction medium consisted of

phosphate buffer (pH 8.0), 10 mmol l^{-1} EDTA, 0.25 mmol l^{-1} GSSG and 0.1 mmol l^{-1} NADPH in 238 mmol l^{-1} $NaHCO_3$ at a final concentration [25].

Glutathione-S-transferase (GST) activity was assayed based on the conjugation reaction with GSH, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [26]. Sample aliquots (0.1 ml) were added to 1.2 ml of the assay mixture containing 100 mmol l^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1 mmol l^{-1} each. The activity was calculated from the changes in absorbance at 340 nm. It was expressed as pmol min^{-1} mg $protein^{-1}$ using $\varepsilon_{340} = 9.6$ $mmol^{-1}$ cm^{-1}.

Non-enzymatic Antioxidants

Non-protein thiols (NPSH) represent an indirect measure of GSH. Proteins were eliminated by adding 0.5 mmol l^{-1} perchloric acid to the homogenates and centrifuging the mixture at 700 *g* for 5 min. To the supernatants (0.1 ml), 0.9 ml of 0.2 mol l^{-1} phosphate buffer (pH 8.0) and 0.18 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at a final concentration were added and vortexed. The DTNB formed a yellow complex with GSH, and the absorbance was measured at 412 nm. NPSH content was expressed as nmol mg $protein^{-1}$ using $\varepsilon_{412} = 13.6$ $mmol^{-1}$ cm^{-1} [27].

The ascorbic acid measurement was carried out according to the method of Roe and Kuether [28]. Tissue homogenates were prepared in 240 mmol l^{-1} TCA and then centrifuged 700*g* for 5 min. The samples aliquots (0.3 ml) were treated with charcoal and filtered. It was added to 0.1 ml of the assay mixture containing 0.15 mol l^{-1} thiourea and 89 mmol l^{-1} DNPH at a final concentration. It was incubated at 37 °C for 3 h. Thereafter, color was produced by adding 0.5 ml of 16 mol l^{-1} H_2SO_4. The absorbance was read at 540 nm. The standard curve was prepared by using different ascorbic acid concentrations (0.005–0.05 mg ml^{-1}) and the slope was used to express the amount of ascorbic acid as μM mg $protein^{-1}$.

Total Reactive Antioxidant Potential

Total reactive antioxidant potential (TRAP) was measured based on the capacity of the sample to scavenge luminol luminescence induced by thermolysis of 2,2′-azo-bis(2-amidinopropane) (ABAP). ABAP is a source of free radicals that react with luminol, yielding chemiluminescence. The addition of sample aliquots (0.02 ml) to 0.28 ml of the reaction medium consisting of 50 mmol l^{-1} phosphate buffer (pH 7.4), 2 mmol l^{-1} ABAP, 1.42 μmol l^{-1} luminol at a final concentration decreases chemiluminescence to basal levels for a period proportional to the total load of antioxidants present in the brain. The standard curve was prepared by using different Trolox concentrations (1–4 μmol l^{-1}). The comparison of the induction time after the addition of Trolox and the brain sample allowed calculation of TRAP as the equivalent of the Trolox concentration necessary to produce the same induction time [29]. The results were expressed as μmol mg $protein^{-1}$.

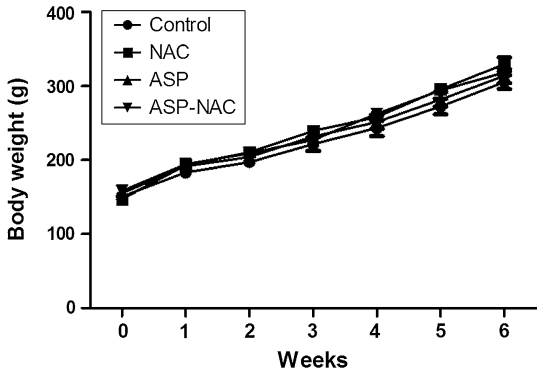


Fig. 1 Effects of *N*-acetylcysteine on the body weight of control and rats chronically treated with aspartame. Values are expressed as mean ± standard error (n = 10)

Protein Assay

Protein was determined by the method of Lowry et al. [30] by using bovine serum albumin as standard.

Statistical Analysis

The statistical analysis was performed by using the software Statistica® 7.0. Levene's test was used to verify whether the data were parametric. Two-way analysis of variance followed by Duncan's test was performed to assess the differences among the groups. The results are expressed as mean ± standard error. The minimum significance level was set at 95 % ($P < 0.05$).

Results

Body Weight

The body weight of the rats did not vary across the different groups during the six experimental weeks ($P > 0.05$) (Fig. 1).

Blood Analysis

The glucose levels were 1.6-fold higher in the ASP than in the control group ($P < 0.05$). Interestingly, in the ASP–NAC group, glucose levels remained increased, being not only 1.5-fold higher when compared to the control group ($P < 0.05$), but also 1.4-fold higher than in the NAC ($P < 0.05$) (Fig. 2).

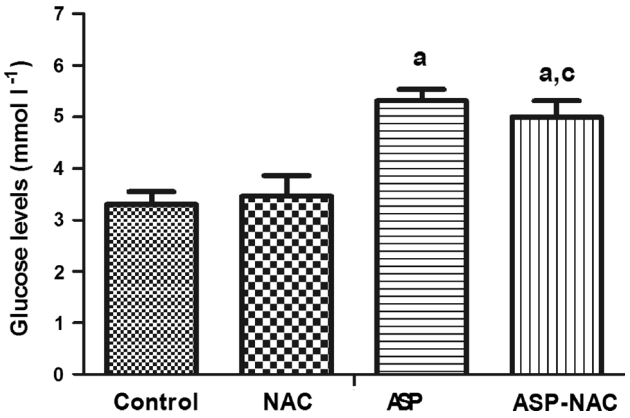


Fig. 2 Effects of *N*-acetylcysteine on the glucose levels of control and rats chronically treated with aspartame. Each *bar* represents the mean ± standard error (n = 10). [a]Denotes that the data are significantly different from the control group at $P < 0.05$. [c]Denotes that the data are significantly different from NAC at $P < 0.05$

Oxidative Stress Biomarkers

The TBARS (Fig. 3a), LOOH (Fig. 3b) and carbonyl protein (Fig. 3c) levels were higher in ASP than in control group ($P < 0.05$). The ASP–NAC animals showed a lower TBARS (Fig. 3a), LOOH (Fig. 3b) and carbonyl protein (Fig. 3c) levels when compared to ASP ($P < 0.05$). The SOD activity was lower in ASP than in the control group ($P < 0.05$). The ASP–NAC showed a higher SOD activity when compared to ASP ($P < 0.05$) (Fig. 4a). The CAT activity was lower in ASP than in the control group ($P < 0.05$). The ASP–NAC animals showed a lower CAT activity when compared to both NAC and control group ($P < 0.05$) (Fig. 4b). The GPx activity was lower in ASP than in the control group ($P < 0.05$). The ASP–NAC animals showed a higher GPx activity when compared to ASP ($P < 0.05$) (Fig. 4c). The GR activity was lower in ASP than in the control group ($P < 0.05$). The ASP–NAC group showed a higher GR activity when compared to ASP ($P < 0.05$) (Fig. 4d). The GST activity was lower in ASP than in the control group ($P < 0.05$). The ASP–NAC rats showed a lower GST activity when compared to both NAC and control group ($P < 0.05$) (Fig. 4e). The NPSH levels were lower in ASP than in the control group ($P < 0.05$). The ASP–NAC group showed a higher NPSH levels when compared to ASP ($P < 0.05$) (Fig. 4f). The ascorbic acid content did not vary across the different experimental groups ($P > 0.05$) (Fig. 4g). The TRAP levels were higher in ASP than in the control group ($P < 0.05$). The ASP–NAC rats showed a higher TRAP levels when compared to ASP ($P < 0.05$) (Fig. 4h).

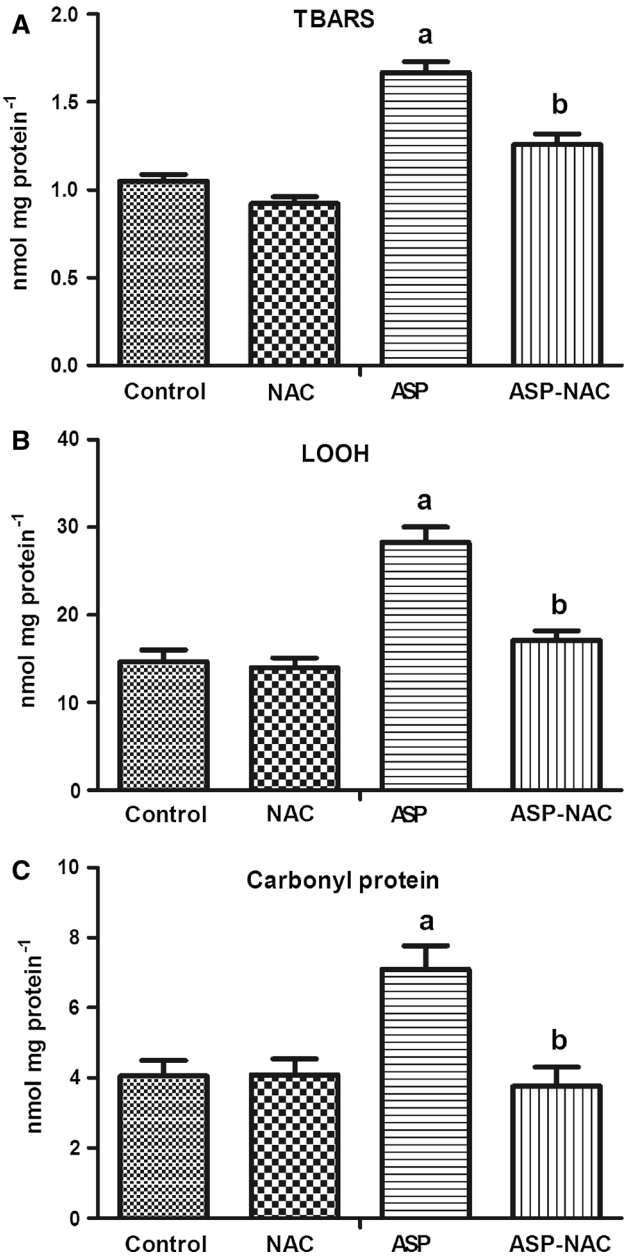


Fig. 3 Effects of *N*-acetylcysteine on TBARS (**a**), LOOH (**b**), and carbonyl protein (**c**) levels in the brain of control and rats chronically treated with aspartame. Each *bar* represents the mean ± standard error (n = 10). [a]Denotes that the data are significantly different from the control group at $P < 0.05$. [b]Denotes that the data are significantly different from ASP at $P < 0.05$

Discussion

Aspartame is a low-calorie sweetener that is mainly used by people trying to lose weight or patients with diabetes mellitus [1]. However, hyperglycemia was previously reported in the literature after chronic exposure to aspartame in hypercholesterolemic zebra fish [31] and in healthy young adult mice [32]. In accordance, as our data, the long-

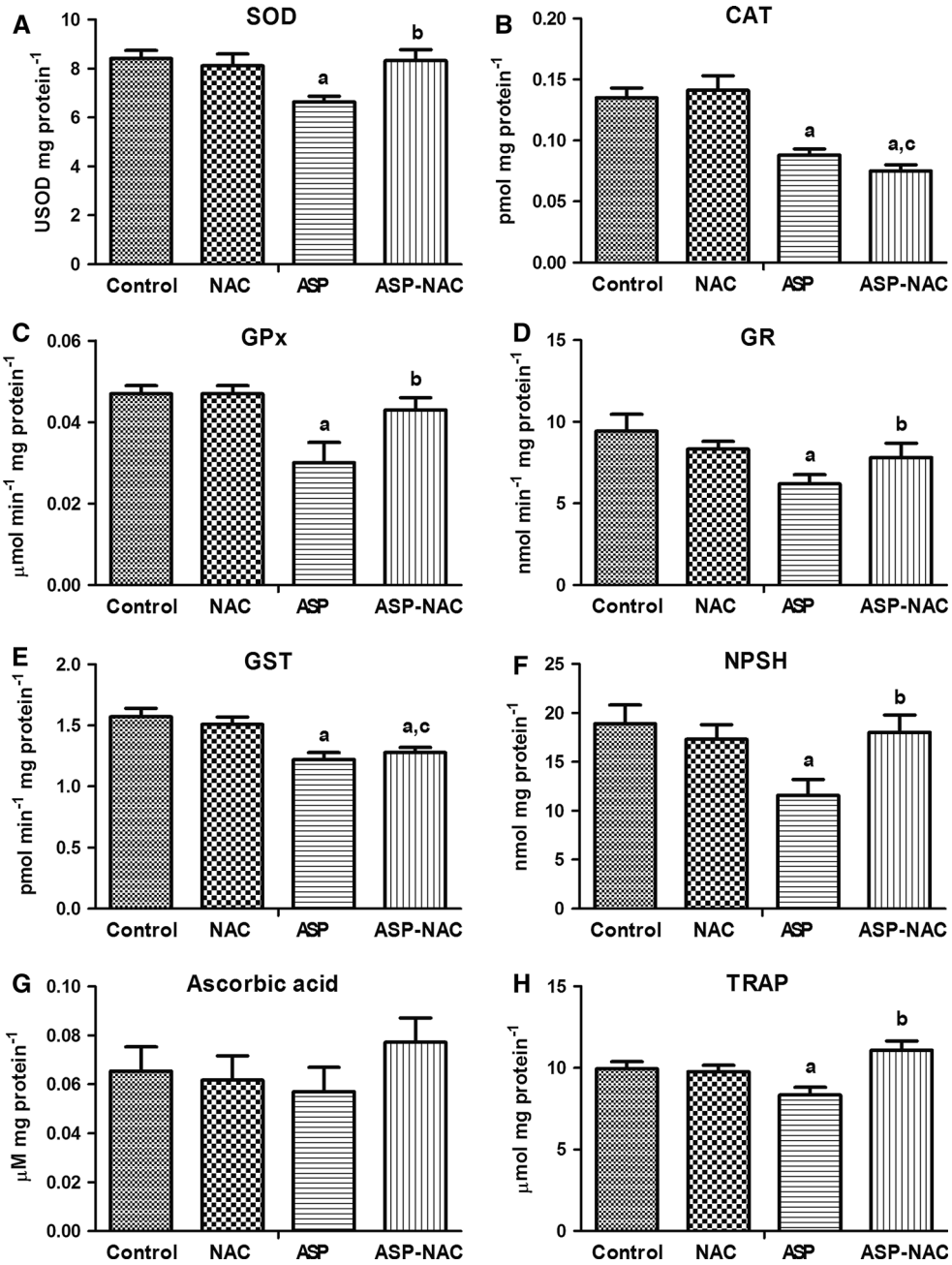
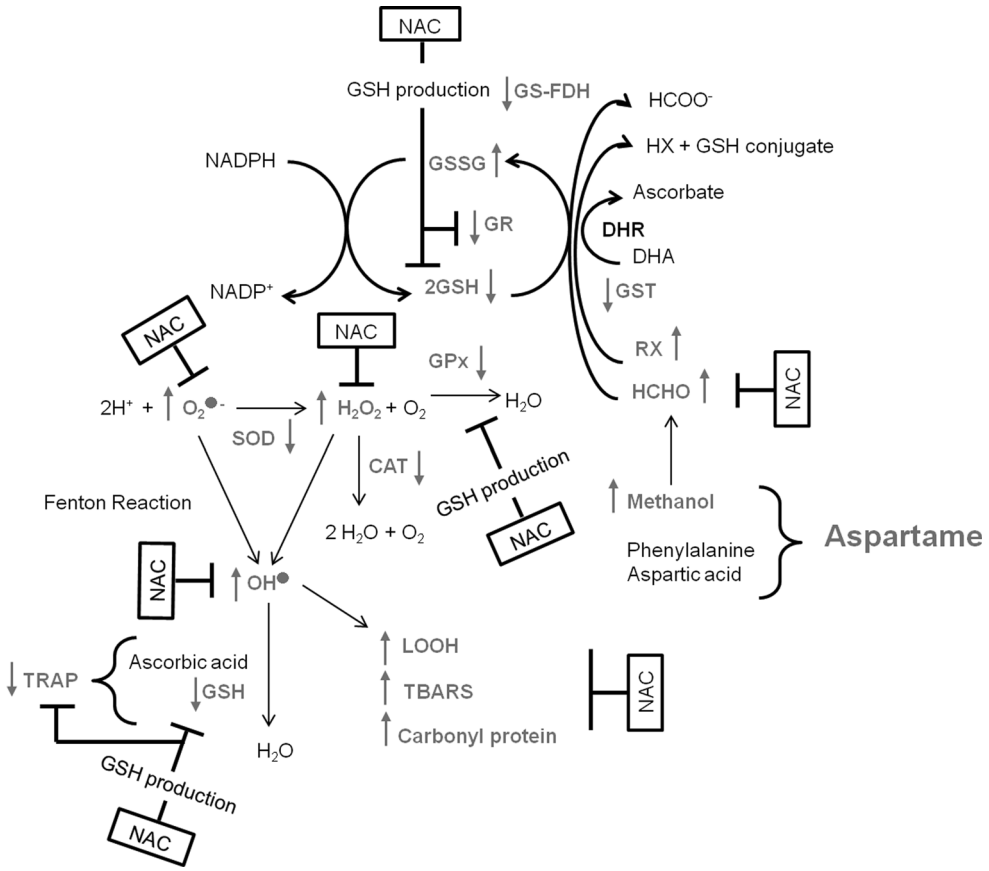


Fig. 4 Effects of *N*-acetylcysteine on SOD (**a**), CAT (**b**), GPx (**c**), GR (**d**), GST (**e**) activities, and NPSH content (**f**), ascorbic acid (**g**), and TRAP (**h**) levels in the brain of control and rats chronically treated with aspartame. Each *bar* represents the mean ± standard error (n = 10). [a]Denotes that the data are significantly different from the control group at $P < 0.05$. [b]Denotes that the data are significantly different from ASP at $P < 0.05$. [c]Denotes that the data are significantly different from NAC at $P < 0.05$

term treatment with aspartame resulted in a mild state of hyperglycemia even after *N*-acetylcysteine administration, indicating that such increase of the serum glucose levels was not triggered by the aspartame-induced oxidative stress. Collison et al. [32] indicated that, in addition to elevate the fasting blood glucose levels, the exposure to artificial sweeteners, including aspartame, might promote impairment in insulin sensitivity, which could result of the increase in the cephalic phase of insulin secretion evoked by the recognition of their sweet taste [33]. Moreover, it was shown that phenylalanine, a metabolite of aspartame, might stimulate an increase in insulin and glucagon levels in serum of healthy individuals [34] and induce an elevation in hepatic glucose production and glucose levels in plasma of rats [35]. Further evidence for a mechanism involved in the effects of aspartame on glucose homeostasis is that its chronic consumption can lead to an increase in the muscarinic receptor density by up to 80 % in many areas of the rat brain [36], which, when activated can contributes to the elevation of plasma glucose levels [37]. The time of exposure to aspartame appears to be critical to such outcomes, since its acute administration to adult diabetic rats had no effect on the serum glucose levels [38]. Abnormal glucose homeostasis and oxidative stress



Scheme 1 The protective mechanism of the *N*-acetylcysteine (NAC) on the aspartame-induced oxidative stress in the rat brain. Aspartame is metabolized to aspartate, phenylalanine, and methanol. Methanol is transformed into formaldehyde (HCHO) and then, through the action of the glutathione-dependent formaldehyde dehydrogenase (GS-FDH), into formate ($HCOO^-$). Therefore, the aspartame consumption caused an increase in the methanol levels, leading to HCHO accumulation and reduced glutathione (GSH) depletion due to the rapid reaction of GSH with HCHO, thereby, reducing HCHO metabolism and elevating its toxicity. Furthermore, the decrease in the GSH levels also occurred because of the reduction in the GR activity, resulting in the increased oxidized glutathione (GSSG) formation and the reduction of total reactive antioxidant potential (TRAP). Since glutathione peroxidase (GPx) and glutathione S-transferase (GST) are enzymes that employ GSH as substrate, the diminution in its levels led to a reduction in their enzymatic activities, occasioning the accumulation of the H_2O_2 and the xenobiotic (RX) (as methanol and its metabolites), respectively. On the other hand, the H_2O_2 accumulation triggered superoxide dismutase (SOD), catalase (CAT) and GPx inactivation, inducing the overproduction of other reactive oxygen species (ROS), as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), which caused oxidative damage to the cell membrane, as observed through the elevation of lipid hydroperoxides (LOOH), thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels. NAC performed a protective effect through the GSH production, thus, re-establishing GPx and GR activities, and the TRAP levels, as well as its direct reaction with the ROS and the HCHO, hence, diminishing their toxicity to the cell membrane. Aspartame-induced changes are represented by the gray color. *DHA* Dehydroascorbic acid, *DHR* dehydroascorbate reductase, H^+ hydrogen ion, H_2O water, *GSX* glutathione S-conjugate, $NADP^+$ nicotinamide adenine dinucleotide phosphate, *NADPH* nicotinamide adenine dinucleotide phosphate reduced, O_2 molecular oxygen

were also linked to memory loss in mice after chronic treatment with aspartame [12]. For the first time, our study provides the support that *N*-acetylcysteine treatment was able to prevent the oxidative stress in the brain of the rats chronically exposed to aspartame.

The brain is rich in polyunsaturated fatty acid and contains relatively low antioxidant capacity [39]. It has been shown that aspartame and its metabolite methanol trigger lipid peroxidation in the rodent brain [12, 15] and its several regions [11, 14] through the ROS formation [40], resulting in loss of its structural and functional cell function. Beyond showing that aspartame occasioned protein carbonylation, we also exhibited that it is able to initiate and terminate the lipid peroxidation, since LOOH are the first byproducts of the lipid peroxidation and degrade into different cytotoxic aldehydes, such as malondialdehyde, which were detected using TBARS [41].

GSH is a non-enzymatic antioxidant that plays a critical role in cellular defense. It is found in greater concentration in neurons [42]. However, its deficit in the brain of rats [11] and in different brain regions of mice [12, 14] was shown to be associated in a unanimous manner to the aspartame

consumption due to its involvement in the methanol metabolism, which depends on GSH. In accordance, we related a decrease in the NPSH levels, which could be linked to the reduced activity of GR due to the direct action of methanol and the LOOH formed during methanol intoxication, as reported by Skrzydlewska et al. [43]. The GR utilizes the reducing equivalents of NADPH [38] for recycle back GSSG to GSH molecules; therefore, we proposed that the decline in the GR activity might result in GSSG accumulation and the reduction in the TRAP, since GSH is the most abundant intracellular non-enzymatic antioxidant [42].

Additionally, GSH is employed as a substrate for GST and GPx activities, thus, we believe that its insufficiency might cause a diminution in their activities. GST constitutes a family of phase II detoxification enzymes involved in the detoxification of xenobiotic and in the signaling cascades [26], whilst GPx converts H_2O_2 and organic hydroperoxides to less reactive products [44]. Hence, we hypothesized that the decrease in the GST activity might result in xenobiotic accretion; whereas the decrease in the GPx activity might occasion the H_2O_2 accumulation and a further inactivation in its activity, as well as an inactivation in the CAT and SOD activities. Consequently, the brain becomes even more susceptible to oxidative processes, suffering lipid peroxidation and protein oxidation through the overproduction of $O_2^{\cdot-}$ and $OH^{\cdot}$, since SOD, CAT, and GPx constitute a mutually supportive team of enzymes that provides defense against ROS. Such enzyme system is essential to dispose of the ROS generated in the brain because CAT and GPx protect SOD against inactivation by hydrogen peroxide (H_2O_2), while in a reciprocal manner SOD protects CAT and GPx against superoxide anion ($O_2^{\cdot-}$) [45].

N-Acetylcysteine treatment prevented all aspartame-induced oxidative injuries possibly due to its reaction with ROS and formaldehyde, diminishing their toxicity; as well as acting as a donor of sulfhydryl groups, stabilizing the membrane lipid-protein structure, as previously reported by Farbiszewski et al. [16]. These authors showed that *N*-acetylcysteine administration inhibited the lipid peroxidation products measured by TBARS, whose levels were increased in the rat brain after methanol treatment. Likewise, we verified that, with the exception of the CAT and GST, *N*-acetylcysteine usage performed a protective effect on the enzymatic antioxidant system of aspartame-treated rats. Jain et al. [46] described that GPx is more important than CAT in destroying H_2O_2 in the brain; consequently, we suggested that even in the absence of an adequate CAT activity to degrade H_2O_2, GPx seemed to be able to protect the brain against further oxidative damage by removing H_2O_2· Moreover, although the literature affirms that *N*-acetylcysteine can mildly stimulates detoxifying phase II enzymes [47], we believe that the exhaustion of the GST activity in aspartame-exposed animals occurred in an attempt to detoxify the brain against aspartame toxic effects, since it plays an important role in the detoxification and metabolism of many xenobiotic compounds [48].

The uniqueness of *N*-acetylcysteine is most probably due to its serving as a precursor of L-cysteine for GSH synthesis [17]. It was shown that L-cysteine and GSH restored the membrane Na^+/K^+ ATPase, whose activity was inhibited as a result of the exposure to aspartame or its metabolites corresponding to the intake of common, abusive, or toxic doses (34, 150, or 200 mg kg^{-1}, respectively), in the rat hippocampus [49] and in the cerebral cortex [40] as result of the amelioration of the cellular GSH reduction from the action of methanol or by their scavenging effect on numerous ROS. Hence, we supposed that *N*-acetylcysteine acted as a protective compound in the brain of aspartame-treated rats through the GSH production, and thereby re-establishing GPx and GR activities, and TRAP levels. Meister [50] affirmed that GSH counteracts oxidative stress by providing reducing power to antioxidant enzymes, such as GPx or by directly conjugating oxidants. Likewise, we believe that its direct reaction with the ROS and the formaldehyde caused the increase in SOD and GPx activities, leading to lower susceptibility of the brain these oxidants, as also stated by Farbiszewski et al. [16] after the usage of *N*-acetylcysteine during methanol intoxication.

In conclusion, the long-term ingestion of aspartame by rats at the acceptable daily dose may induce oxidative stress their brain. *N*-Acetylcysteine treatment may ameliorate the toxic effects to proteins and lipids that were induced by aspartame in the brain. These therapeutic effects may be linked to the elevation of NPSH levels and the modulation of the GSH-related enzyme activities (Scheme 1).

Acknowledgments The authors are grateful to the Conselho Nacional de Desenvolvimento Tecnológico (CNPq), to the Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to the Fundo de Incentivo a Pesquisa da Universidade Federal de Santa Maria (FIPE-UFSM).

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