Guanidinoacetate alters antioxidant defenses and butyrylcholinesterase activity in the blood of rats

Guilherme André Eger[1], Vinícius Vialle Ferreira[1],
Camila Ribeiro Batista[1], Daniela Delwing de Lima[2],
Angela Terezinha de Souza Wyse[3], Júlia Niehues da Cruz[4],
Débora Delwing Dal Magro[5], José Geraldo Pereira da Cruz[5]

ABSTRACT

Introduction: Deficiency of guanidinoacetate methyltransferase, the first described creatine biosynthesis defect, leads to depletion of creatine and phosphocreatine, and accumulation of guanidinoacetate (GAA) in brain and body fluids. The present study aimed to investigate the influence of GAA on the activities of antioxidant enzymes, as well as on thiobarbituric acid-reactive substances (TBARS) and butyrylcholinesterase (BuChE) activity in the blood of rats. We also evaluated the effect of trolox (6-hydr oxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), GSH (glutathione) and L-NAME (NG-nitro-L-arginine methyl ester) on the alterations elicited by GAA.

Methods: The rats were randomly divided into 8 groups: (1) control; (2) GAA (10, 30, 50, 100 mM/kg); (3) trolox (1 mM/kg) + control; (4) trolox (1 mM/kg) + GAA (100 mM/kg); (5) GSH (1 mM/kg) + control; (6) GSH (1 mM/kg) + GAA (100 mM/kg); (7) L-NAME (1 mM/kg) + control; (8) L-NAME + GAA (100 mM/kg). After the addition of compounds, erythrocytes and plasma were pre-incubated at 37°C for 1h and tested immediately.

Results: GAA enhanced the activities of catalase (CAT) and glutathione peroxidase (GSH-Px) in the erythrocytes and BuChE activity. In addition, GAA enhanced TBARS levels in the plasma. Trolox, GSH and L-NAME addition prevented the majority of alterations in oxidative stress parameters and the increase of BuChE activity that were caused by GAA. Data suggest that GAA alters antioxidant defenses and induces lipid peroxidation in the blood, as well altering BuChE activity. However, in the presence of trolox, GSH and L-NAME some of these alterations in oxidative stress and BuChE activity were prevented.

Conclusions: Our findings lend support to a potential therapeutic strategy for this condition, which may include the use of appropriate antioxidants for ameliorating the damage caused by GAA.

Keywords: Butyrylcholinesterase; glutathione; guanidinoacetate; L-NAME; TBARS; trolox

1 Department of Medicine, Regional University of Blumenau. Blumenau, SC, Brazil.

2 Department of Medicine, University of Joinville Region, Campus Universitário. Joinville, SC, Brazil.

3 Department of Biochemistry, Federal University of Rio Grande do Sul. Porto Alegre, RS, Brazil.

4 Medical Clinic, Hans Dieter Schmidt Hospital. Joinville, SC, Brazil.

5 Department of Natural Sciences, Regional University of Blumenau. Blumenau, SC, Brazil.

Corresponding author:
José Geraldo Pereira da Cruz
E-mail: cruzjgp@gmail.com
Departamento de Ciências Natural,
Universidade Regional de Blumenau
Rua Antônio da Veiga, 140.
89012-900, Blumenau, SC, Brazil.

Guanidinoacetate methyltransferase (GAMT) deficiency is a creatine synthesis disorder characterized by cerebral creatine deficiency and an accumulation of GAA in tissues and body fluids[1]. Affected individuals exhibit marked impairment of expressive speech, autistic features, and varying neurological manifestations, including epilepsy and movement disorders[2,3]. The creatine, phosphocreatine and creatine kinase system play essential roles to maintain the high energy levels necessary for brain development and functions, through regeneration and buffering of ATP levels[4]. Additionally, other researchers suggest that creatine in central nervous system may also

act as true neurotransmitter and one of the main central nervous system osmolytes[5-7].

We have previously demonstrated that GAA induced lipid peroxidation in the brain[8]. These data are in agreement with the other studies, showing that GAA increases free radical production[9-11]. It is therefore presumed that this pathomechanism may contribute at least in part to the pathophysiology of the brain injury observed in patients affected by GAMT deficiency. Lipid peroxidation, the oxidative catabolism of polyunsaturated fatty acids, is widely accepted as a general mechanism for cellular injury and death, and has been implicated in diverse pathological conditions[12,13]. SOD, CAT, and GSH-Px are endogenous antioxidant enzymes that act as free-radical scavengers and hence prevent and repair damage done by reactive oxygen species[14,15]. In addition, acetylcholinesterase activity is significantly increased by GAA in the rat striatum, interfering in acetylcholine levels; we also verified the *in vitro* effects of different concentrations of GAA on BuChE activity in the serum of rats[10,16].

Considering the absence of studies on GAA in the peripheral system, the purpose of this study was to investigate the *in vitro* effects of different concentrations of GAA on the activities of antioxidant enzymes CAT, GSH-Px, and SOD, as well as on TBARS in the blood and BuChE activity in the plasma of rats. Furthermore, we also tested the influence of trolox, GSH and L-NAME on the effects elicited by GAA on antioxidant enzymes, TBARS, and on BuChE activity.

METHODS

Subjects

Sixty-day-old normal Wistar rats obtained from the Central Animal House of the Regional University of Blumenau, Blumenau, state of Santa Catarina, Brazil, were used in the experiments. The animals from our own breeding stock were maintained on a 12-h light/12-h dark cycle at a constant temperature (22±1°C), with free access to water and commercial protein chow. The experiments were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior, which are based on the United States National Institutes of Health *Guide for Care and Use of Laboratory Animals*. All experiments were approved by the local ethical committee of Regional University of Blumenau-SC, Brazil (Protocol 010/12).

Drugs Administration

The assays were divided into eight groups; group 1 (saline), group 2 (GAA 10 µM, 30 µM, 50 µM and 100 µM), group 3 (1.0 mM trolox), group 4 (GAA + 1.0 mM trolox), group 5 (1.0 mM GSH), group 6 (GAA + 1.0 mM GSH), group 7 (1.0 mM L-NAME) and group 8 (GAA + 1.0 mM L-NAME). After the addition of compounds, erythrocytes or plasma were pre-incubated at 37°C for 1 h. The doses of trolox, GSH and L-NAME utilized were chosen according to Wyse et al.[17], Avrova et al.[18] and Qi et al.[19].

Erythrocyte and Plasma Preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from rats. Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1.000 × g, plasma was then removed by aspiration and frozen at –80°C until use in assays. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 mL of distilled water to 100 µL of washed erythrocytes and frozen at −80°C until determination of the antioxidant enzyme activities. For antioxidant enzyme activity determination, erythrocytes were frozen and thaw three times, and centrifuged at 13.500 × g for 10 min. The supernatant was diluted in order to achieve an approximate concentration of 0.5 mg/mL of protein.

Catalase Assay (CAT)

CAT activity was assayed by the method of Aebi[20]. Hydrogen peroxide (H_2O_2) disappearance was continuously monitored with a spectrophotometer at 240 nm for 90 s. One unit of the enzyme is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

Glutathione Peroxidase Assay (GSH-Px)

GSH-Px activity was measured by the method of Wendel[21]. However, the concentration of NADPH was adjusted to 0,1 mM, after previous tests performed in our laboratory. Tert-butyl hydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GSH-Px unit is defined as 1 µmol of NADPH consumed per minute and specific activity is reported as units per mg protein.

Superoxide Dismutase assay (SOD)

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2, which is a substrate for SOD[22]. The inhibition of the autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units/mg protein.

Thiobarbituric acid reactive substances (TBARS)

TBARS was determined according to the method described by Esterbauer and Cheeseman[23]. TBARS measures malondialdehyde, a product of lipoperoxidation caused mainly by hydroxyl free radicals. For measurements, plasma was mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid and heated in a boiling water bath for 25 min. TBARS was determined by the absorbance at 535 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the supernatants. TBARS was calculated as nanomole of malondialdehyde formed per milligram of protein.

BuChE activity assay

Butyrylcholinesterase activity was determined by the method of Ellman et al. with some modifications[24]. The hydrolysis rate was measured at an acetylthiocholine concentration of 0.8 mM in 1 mL assay solutions with 100 mM phosphate buffer, pH 7.5 and 1.0 mM 5,5'-dithiobis-2-nitrobenzoic acid. Fifty microliters of rat plasma were added to the reaction mixture and pre-incubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of 5.5'-dithiobis-

2-nitrobenzoic acid at 412 nm for 2-3 min (intervals of 30 s) at 25°C.

Protein Determination

Protein was measured by the Lowry et al., using serum bovine albumin as standard[25].

Statistical Analysis

Data were analyzed by ANOVA followed by the Duncan multiple range test when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $p < 0.05$ were considered to be significant.
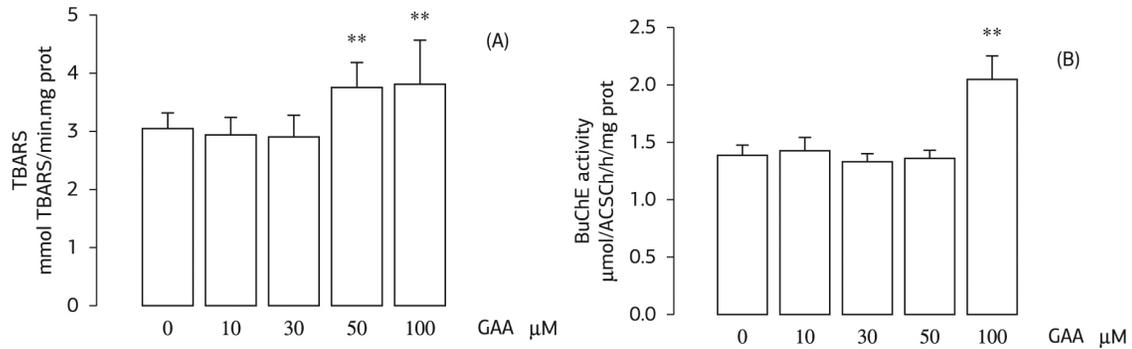
RESULTS

Figure 1 shows that GAA, at a concentration of 100 µM, significantly enhanced CAT (F = 19.751; $p < 0.001$, figure 1A) and GSH-Px (F = 8.217; $p < 0.001$, figure 1B) activities in the erythrocytes of rats, as compared to control groups. On the other hand, Figure 1C shows that GAA did not alter SOD activity at any concentration studied (F = 0.356; $p > 0.05$) in the erythrocytes of rats, as compared to the control group.

Figure 2 A shows that GAA, at a concentration of 100 µM, significantly enhanced TBARS levels (F = 6.283; $p < 0.001$). Figure 2B shows that GAA, at a concentration of 100 µM, enhanced BuChE activity (F = 40.915; $p < 0.001$) in the plasma of rats, as compared to control groups.

As can be observed in Figure 3A, B, and C, respectively; trolox, GSH and L-NAME, were able to prevent the increase in CAT activity in the erythrocytes of rats (F = 32.739; $p < 0.001$). GSH and L-NAME, but not trolox, were able to prevent the increase in GSH-Px activity (F = 22.286; $p < 0.001$), while trolox and L-NAME prevented the increase in TBARS levels caused by GAA at concentrations of 100 µM,
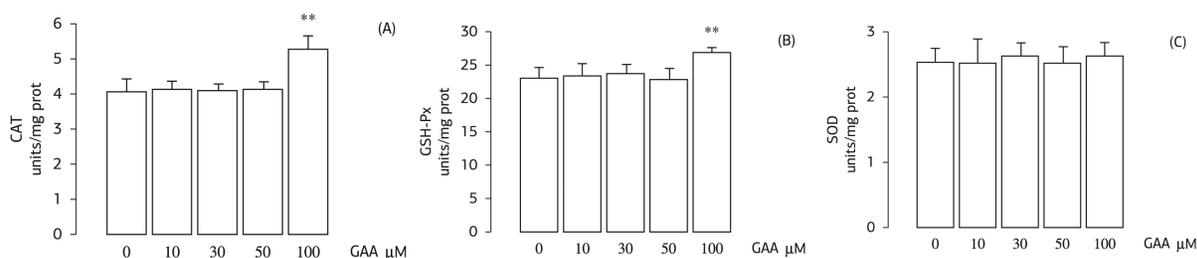
Figure 1: *In vitro* effects of increasing concentrations of guanidinoacetate (GAA) on antioxidant enzyme activities in rat erythrocytes. Data are mean ± SD (n = 7). **$p < 0.001$; compared to control group (Duncan's multiple range test).

Figure 2: *In vitro* effects of increasing concentrations of guanidinoacetate (GAA) on thiobarbituric acid reactive substances (TBARS – 2 A) and on butyrylcholinesterase (BuChE – 2 B) activity in the plasma of rats. Results are expressed as mean ± SD (n = 7). **p<0.001; compared to control group (Duncan's multiple range test).
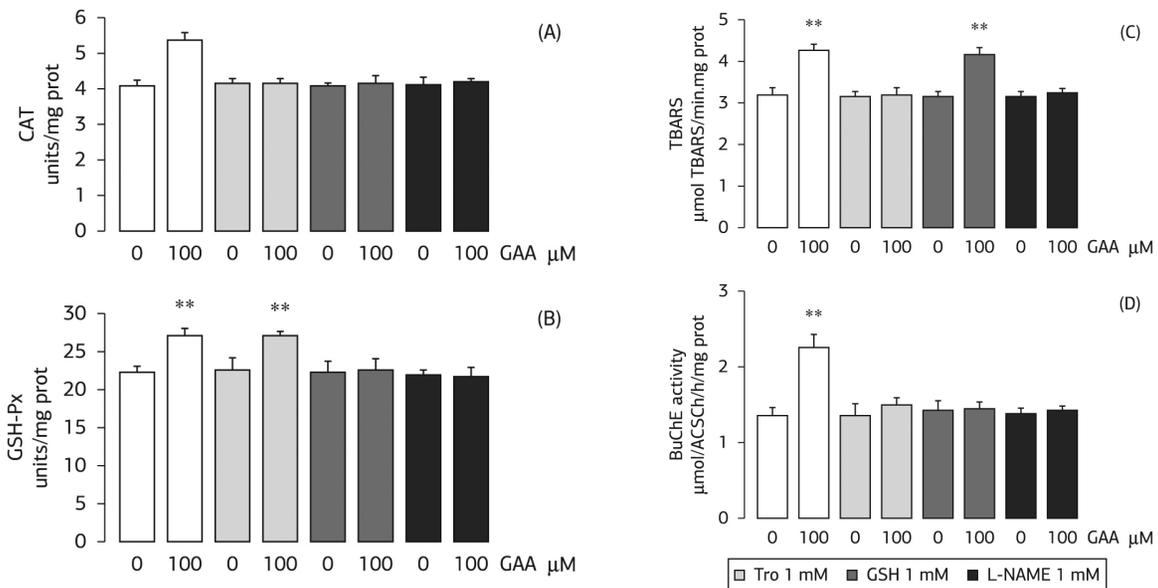
Figure 3: *In vitro* effects of trolox (Tro), glutathione (GSH) and L-NAME on CAT (A), GSH-Px (B) and TBARS (C) in the erythrocytes and and on butyrylcholinesterase (BuChE) activity (D) in plasma of rats in the presence or absence of guanidinoacetate (GAA). Data are mean ± SD (n = 6). **p<0.001; compared to control group (Duncan's multiple range test).

but not GSH (F = 70.252; p<0.001), as compared to controls. With respect to BuChE activity, Figure 3D shows that trolox, GSH and L-NAME were able to prevent the increase in BuChE activity in the plasma of rats (F = 36.686; p<0.001).

DISCUSSION

Results showed that GAA significantly enhanced CAT and GSH-Px activities in the erythrocytes of rats, as compared to these activities in the control groups. On the other hand, GAA did not alter SOD activity, at any concentration studied, in the erythrocytes of rats, as compared to the control group. Regarding TBARS levels, GAA significantly enhanced TBARS

levels in the plasma of rats. Although we cannot precisely establish the mechanisms by which GAA administration caused the increases in CAT and GSH-Px activities in the erythrocytes, it is possible that this occurred due to the increased generation of free radicals, provoked by GAA. Previous data have shown that antioxidant enzymes may respond to oxidative stress by increasing their activity in order to reduce damage[14]. Oxidative stress is commonly observed in some inborn errors of intermediary metabolism[14]. Although the cause of increased oxidative stress in these diseases is not completely understood, it may be due to the accumulation of toxic metabolites that lead to excessive production of free radicals. Oxidative stress may also occur due to a substantial increase

in metabolic by-products that directly or indirectly deplete the cells antioxidant capacity. In this context, previous studies have shown that the addition of GAA to assays (*in vitro* studies) significantly decreased total radical-trapping antioxidant potential, SOD activity, and total thiol levels in rat striatum[11].

Results have shown that GAA enhances BuChE activity in the plasma of rats, reducing acetylcholine levels. Although the exact physiological function of BuChE is unclear, it has been shown that it can substitute acetylcholinesterase in maintaining the structural and functional integrity of cholinergic pathways[16]. It has also been shown that acetylcholine has a potential neuroprotective role as a scavenger of superoxide anion and is able to reduce lipid peroxidation, which suggests that a decrease in acetylcholine levels could result in a decrease in neuroprotection that can lead to neurodegeneration[26]. Our results point to a similar protective role by acetylcholine in the blood.

Finally, we also evaluated the influence of trolox, GSH and L-NAME on the effects elicited by GAA, in order to investigate the possible participation of free radicals in the effects of GAA on CAT, GSH-Px, BuChE activities and on TBARS. *Post hoc* analyses showed that trolox, GSH and L-NAME *per se* did not alter these parameters. Results showed that trolox, GSH and L-NAME, were able to prevent the increase in CAT activity in the erythrocytes of rats. GSH and L-NAME, but not trolox, were able to prevent the increase in GSH-Px activity in the erythrocytes of rats, while trolox and L-NAME prevented the increase in TBARS levels caused by GAA. With regard to BuChE activity, data showed that trolox, GSH and L-NAME were able to prevent the increase in BuChE activity in the plasma of rats. In this context, trolox interacts with cell membranes, traps free radicals and interrupts the chain of oxidative reactions that damage cells, L-NAME is a potent nitric oxide synthase inhibitor and GSH acts as an SH-group protecting agent[26-28]. Results suggest that the scavenging of free radicals by trolox, and/or nitric oxide and the oxidation of SH groups are involved in the effects of GAA on the enzyme activities. In addition, the scavenging of free radicals by trolox and nitric oxide are involved in the effects of GAA on TBARS levels. Nitric oxide, however, is also a free radical and hence in many biological systems it has a short half-life due to its reactivity with other intracellular constituents, such as superoxide. The reaction between nitric oxide and superoxide results in the formation of the peroxynitrite anion, which is extremely cytotoxic. Despite the important physiological roles attributed to nitric oxide, excessive generation of this molecule and also of its derivate anion have been implicated in the pathophysiology of common conditions such as stroke, Alzheimer's and Parkinson diseases, cardiovascular disease, atherogenesis and the atherosclerotic state[29].

The present study reinforces the hypothesis that oxidative stress is induced in GAMT-deficiency. Furthermore, our results indicate that GAA increases enzymatic antioxidant defenses, cause lipid oxidation and enhanced BuChE activity, probably by enhancing reactive species in the blood of rats. In addition, we demonstrated that the alterations in these parameters of oxidative stress and BuChE activity are probably mediated by the generation of NO and /or other free radicals, which are scavenged by trolox and GSH, since L-NAME and these antioxidants prevented these effects caused by GAA in the blood of rats. Although it is difficult to extrapolate our findings to human GAMT-deficiency, if that is the case, the present results may be relevant to explain, at least in part, the pathophysiological characteristics present in the affected patients. Further studies are required to determine whether antioxidants should be considered as an adjuvant therapy to specific diets in GAMT-deficiency.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Comeaux MS, Wang J, Wang G, Kleppe S, Zhang VW, Schmitt ES, et al. Biochemical, molecular, and clinical diagnoses of patients with cerebral creatine deficiency syndromes. *Mol Genet Metab*. 2013;109(3):260-8. http://dx.doi.org/10.1016/j.ymgme.2013.04.006. PMid:23660394

2. Nasrallah F, Feki M, Kaabachi N. Creatine and creatine deficiency syndromes: biochemical and clinical aspects. *Pediatr Neurol*. 2010;42(3):163-71. http://dx.doi.org/10.1016/j.pediatrneurol.2009.07.015. PMid:20159424

3. Leuzzi V, Mastrangelo M, Battini R, Cioni G. Inborn errors of creatine metabolism and epilepsy. *Epilepsia*. 2013;54(2):217-27. http://dx.doi.org/10.1111/epi.12020. PMid:23157605

4. Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR. Functions and effects of creatine in the central nervous system. *Brain Res Bull*. 2008;76(4):329-43. http://dx.doi.org/10.1016/j.brainresbull.2008.02.035. PMid:18502307

5. Almeida LS, Salomons GS, Hogenboom F, Jakobs C, Schoffelmeer AN. Exocytotic release of creatine in rat brain. *Synapse*. 2006;60(2):118-23. http://dx.doi.org/10.1002/syn.20280. PMid:16715490

6. Bothwell JH, Styles P, Bhakoo KK. Swelling-activated taurine and creatine effluxes from rat cortical astrocytes are pharmacologically distinct. *J Membr Biol*. 2002;185(2):157-64. http://dx.doi.org/10.1007/s00232-001-0121-2. PMid:11891574

7. Braissant O, Henry H, Béard E, Uldry J. Creatine deficiency syndromes and the importance of creatine synthesis in the brain. *Amino Acids*. 2011;40(5):1315-24. http://dx.doi.org/10.1007/s00726-011-0852-z. PMid:21390529

8. Kolling J, Wyse AT. Creatine prevents the inhibition of energy metabolism and lipid peroxidation in rats subjected to GAA administration. *Metab Brain Dis*. 2010;25(3):331-8. http://dx.doi.org/10.1007/s11011-010-9215-9. PMid:20830606

9. Zugno AI, Scherer EB, Mattos C, Ribeiro CA, Wannmacher CM, Wajner M, et al. Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na+,K+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum in vivo. *Biochim Biophys Acta*. 2007;1772(5):563-9. http://dx.doi.org/10.1016/j.bbadis.2007.02.005. PMid:17407807

10. Zugno AI, Pereira LO, Mattos C, Scherer EB, Netto CA, Wyse AT. Guanidinoacetate administration increases acetylcholinesterase activity in striatum of rats and impairs retention of an inhibitory avoidance task. *Metab Brain Dis*. 2008;23(2):189-98. http://dx.doi.org/10.1007/s11011-008-9085-6. PMid:18437545

11. Zugno AI, Stefanello FM, Scherer EB, Mattos C, Pederzolli CD, Andrade VM, et al. Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats. *Neurochem Res*. 2008;33(9):1804-10. http://dx.doi.org/10.1007/s11064-008-9636-6. PMid:18343996

12. Garcia-Souza LF, Oliveira MF. Mitochondria: biological roles in platelet physiology and pathology. *Int J Biochem Cell Biol*. 2014;50:156-60. http://dx.doi.org/10.1016/j.biocel.2014.02.015. PMid:24569121

13. Maruyama W, Shaomoto-Nagai M, Kato Y, Hisaka S, Osawa T, Naoi M. Role of lipid peroxide in the neurodegenerative disorders. *Subcell Biochem*. 2014;77:127-36. http://dx.doi.org/10.1007/978-94-007-7920-4_11. PMid:24374924

14. Wu JQ, Kosten TR, Zhang XY. Free radicals, antioxidant defense systems, and schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*. 2013;46:200-6. http://dx.doi.org/10.1016/j.pnpbp.2013.02.015. PMid:23470289

15. Wyse AT, Stefanello FM, Chiarani F, Delwing D, Wannmacher CM, Wajner M. Arginine administration decreases cerebral cortex acetylcholinesterase and serum butyrylcholinesterase probably by oxidative stress induction. *Neurochem Res*. 2004;29(2):385-9. http://dx.doi.org/10.1023/B:NERE.0000013741.81436.e8. PMid:15002734

16. Nordberg A, Ballard C, Bullock R, Darreh-Shori T, Somogyi M. A review of butyrylcholinesterase as a therapeutic target in the treatment of Alzheimer's disease. *Prim Care Companion CNS Disord*. 2013;15(2). PMid:23930233

17. Wyse AT, Zugno AI, Streck EL, Matté C, Calcagnotto T, Wannmacher CM, et al. Inhibition of Na(+),K(+)-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. *Neurochem Res*. 2002;27(12):1685-9. http://dx.doi.org/10.1023/A:1021647329937. PMid:12515323

18. Avrova NF, Shestak KI, Zakharova IO, Sokolova TV, Leont'ev VG. The difference in the effect of glutamate and NO synthase inhibitor on free calcium concentration and Na+, K+-ATPase activity in synaptosomes from various brain regions. *Neurochem Res*. 1999;24(9):1101-6. http://dx.doi.org/10.1023/A:1020752101736. PMid:10485580

19. Qi B, Yamagami T, Naruse Y, Sokejima S, Kagamimori S. Effects of taurine on depletion of erythrocyte membrane Na-K ATPase activity due to ozone exposure or cholesterol enrichment. *J Nutr Sci Vitaminol (Tokyo)*. 1995;41(6):627-34. http://dx.doi.org/10.3177/jnsv.41.627. PMid:8926535

20. Aebi H. Catalase in vitro. *Methods Enzymol*. 1984;105:121-6. http://dx.doi.org/10.1016/S0076-6879(84)05016-3. PMid:6727660

21. Wendel A. Glutathione peroxidase. *Methods Enzymol*. 1981;77:325-33. http://dx.doi.org/10.1016/S0076-6879(81)77046-0. PMid:7329310

22. Flohé L, Otting F. Superoxide dismutase assays. *Methods Enzymol*. 1984;105:93-104. http://dx.doi.org/10.1016/S0076-6879(84)05013-8. PMid:6328209

23. Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol*. 1990;186:407-21. http://dx.doi.org/10.1016/0076-6879(90)86134-H. PMid:2233308

24. Ellman GL, Courtney KD, Andres V JR, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961;7(2):88-95. http://dx.doi.org/10.1016/0006-2952(61)90145-9. PMid:13726518

25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-75. PMid:14907713.

26. Bai H, Liu R, Chen HL, Zhang W, Wang X, Zhang XD, et al. Enhanced antioxidant effect of caffeic acid phenethyl ester and Trolox in combination against radiation induced-oxidative stress. *Chem Biol Interact*. 2014;207:7-15. http://dx.doi.org/10.1016/j.cbi.2013.10.022. PMid:24211618

27. Lima DD, Delwing F, da Cruz JG, Wyse AT, Delwing-Dal Magro D. Protective effect of antioxidants on blood oxidative stress caused by arginine. *Fundam Clin Pharmacol*. 2012;26(2):250-8. http://dx.doi.org/10.1111/j.1472-8206.2010.00909.x. PMid:21210846

28. Rodrigues AF, Roecker R, Junges GM, de Lima DD, da Cruz JG, Wyse AT, et al. Hypoxanthine induces oxidative stress in kidney of rats: protective effect of vitamins E plus C and allopurinol. *Cell Biochem Funct*. 2014;32(4):387-94. http://dx.doi.org/10.1002/cbf.3029. PMid:24578313

29. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007;87(1):315-424. http://dx.doi.org/10.1152/physrev.00029.2006. PMid:17237348