Universidade Federal do Rio Grande do Sul

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso de Farmácia

Homocysteine decreases glutamate uptake and Na^+,K^+- ATPase activity and increases oxidative stress: prevention by vitamin C

Fernanda Rossatto Machado

Porto Alegre, junho de 2010.

Universidade Federal do Rio Grande do Sul

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso de Farmácia

Homocysteine decreases glutamate uptake and Na^+,K^+- ATPase activity and increases oxidative stress: prevention by vitamin C

Fernanda Rossatto Machado

Trabalho de Conclusão

da Disciplina de Estágio Curricular em Farmácia

Prof[a]. Dr[a]. Angela T. S. Wyse

Orientadora

Porto Alegre, junho de 2010.

Agradecimentos

À minha orientadora, Angela, por toda a amizade e por sempre contribuir tanto com o meu trabalho e com a minha formação.

Aos amigos do laboratório 34 e 36 pela amizade, que apesar de recente, é tão sólida e verdadeira. Em especial as amigas Andréa e Aline por terem me ajudado tanto na execução deste trabalho.

Aos meus pais, Ricardo e Heloisa, por todo o carinho, por terem entendido a minha ausência, por terem me ensinado que o estudo é o caminho para uma vida profissional e pessoal satisfatória, por estarem ao meu lado em todos os momentos em que a companhia de vocês era essencial: AMO VOCÊS!

Ao meu namorado, Marcelo, pelo incentivo, companheirismo e por acreditar e reforçar junto comigo que o estudo é a base de tudo. Pelo amor tão lindo que construímos durante todos estes anos e por ser a melhor companhia em todos os momentos:

TE AMO MUITO!

À minha irmã, meu amor incondicional, e ao meu cunhado que mesmo de longe transmitiram muito carinho e força me fazendo acreditar que tudo daria certo: AMO VOCÊS!

Aos amigos que fiz durante a graduação, que tornaram os meus dias mais alegres, pelo companheirismo, pelos grupos de estudos e principalmente pela amizade que, com certeza, será para sempre!

À Faculdade de Farmácia, ao Departamento de Bioquímica e a Universidade Federal do Rio Grande do Sul pelo ensino de excelência e gratuito.

Resumo

No presente estudo, nós investigamos o efeito da administração crônica de homocisteína sobre a captação de glutamato e a atividade da Na^+,K^+-ATPase, bem como sobre alguns parâmetros de estresse oxidativo (espécies reativas e enzimas antioxidantes como a catalase e superóxido dismutase) em hipocampo de ratos jovens. A influência da vitamina C, um antioxidante clássico, sobre os efeitos mediados pela homocisteína também foi avaliada. Resultados mostraram que a hiper-homocisteinemia crônica diminuiu a captação de glutamato e a atividade da enzima Na^+,K^+-ATPase, aumentou os níveis de espécies reativas e diminuiu as atividades das enzimas catalase e superóxido dismutase. Além disso, a administração concomitante de vitamina C preveniu significativamente as alterações causadas pela homocisteína. De acordo com nossos resultados, parece possível sugerir que a redução na captação de glutamato e na atividade da Na^+,K^+-ATPase pode ser mediada pelo estresse oxidativo, já que a vitamina C preveniu tais efeitos. Acreditamos que esses resultados possam auxiliar na elucidação dos mecanismos pelos quais a homocisteína exerce seus efeitos neurotóxicos e sugerimos a possibilidade de que o tratamento com antioxidantes pode ser benéfico, se associado a terapia clássica, no tratamento de distúrbios neurológicos observados em pacientes homocistinúricos.

Palavras chaves: hiper-homocisteinemia; captação de glutamato; Na^+,K^+-ATPase; vitamina C; hipocampo

Este artigo foi elaborado segundo as normas da revista *Journal of Neural Transmission* apresentadas em anexo.

Homocysteine decreases glutamate uptake and Na^+,K^+- ATPase activity and increases oxidative stress: prevention by vitamin C

Fernanda R. Machado[1], Andréa G. K. Ferreira [1], Aline A. da Cunha [1], Bárbara Tagliari[1], Ben Hur M. Mussulini [1], Suzana Wofchuk [1], Angela T. S. Wyse [1]

[1]Laboratório de Neuroproteção e Doença Metabólica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS, Brazil.

Address reprint requests to: Dr. Angela T. S. Wyse, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Phone 55 51 3308 5573, Fax 55 51 3308 5535, E-mail: wyse@ufrgs.br

Abstract

In this study we investigated the effect of chronic administration of homocysteine on glutamate uptake and activity of Na^+,K^+-ATPase, as well as on some parameters of oxidative stress, such as levels of reactive species and antioxidant enzymes catalase and superoxide dismutase in the hippocampus of young rats. The influence of vitamin C, a classic antioxidant, on the effects mediated by homocysteine was also evaluated. Results showed that chronic hyperhomocysteinemia decreased glutamate uptake and the activities of Na^+,K^+-ATPase, catalase and superoxide dismutase; the levels of reactive species were increased by chronic homocysteine administration. Moreover, concomitant administration of vitamin C significantly prevented the alterations caused by homocysteine. According to our results, it seems possible to suggest that the reduction in glutamate uptake and activity of Na^+,K^+-ATPase may be mediated by oxidative stress, since vitamin C prevented these effects. We believe that these results may help us to elucidate the mechanisms by which homocysteine exerts its neurotoxic effects and suggest the possibility that treatment with antioxidants may be beneficial, if combined with classical therapy, for the treatment of neurological disorders observed in patients with homocystinuria.

Key words: hyperhomocysteinemia; glutamate uptake; Na^+,K^+-ATPase; oxidative stress; vitamin C; hippocampus

INTRODUCTION

Homocysteine (Hcy) is an amino acid generated metabolically by the S-adenosylmethionine (SAM) -dependent transmethylation pathway (Schalinske 2009) and the tissue accumulation of Hcy occurs in homocystinuria, a metabolic disease characterized biochemically by cystathionine b-synthase (CBS, EC 4.2.1.22) deficiency (Mudd et al. 2001). The accumulation of homocysteine is also present in several disorders that affects the central nervous system (CNS) as epilepsy (Herrmann et al. 2007; Sachdev 2004), Parkinson's (Kuhn et al. 1998) and Alzheimer's disease (Gallucci et al. 2004).

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is probably involved in most aspects of normal brain function including cognition, memory and learning (Collingridge et al. 1989; Fonnun 1984; Headley et al. 1990). However, when glutamate is present in high concentrations in the synaptic cleft, it may lead to excitotoxicity, a process corresponding to glutamate receptor overstimulation that subsequently leads to neuronal damage (Danbolt 2001; Mattson et al. 2002). Previous data suggests that Hcy induces neurodegeneration by NMDA receptor overstimulation (Lipton et al. 1997; Zieminska et al. 2006). More recently, we have shown that Hcy decreased the glutamate uptake in parietal cortex of rats, thus causing glutamatergic excitotoxicity (Matté et al. 2009).

Na^+,K^+-ATPase (EC 3.6.1.3), also known as the sodium pump, is a membrane-bound enzyme responsible for generating the membrane potential of the cell through ion exchange, that provides energy for various cellular functions such as the establishment and maintenance of membrane resting potential and pH homeostasis (Sjöström et al. 2007). Studies in our laboratory indicated that acute administration of Hcy decreased the Na^+,K^+-ATPase activity in hippocampus of rats (Wyse et al. 2002). It has been reported that this enzyme is inhibited by free radicals (Hitschke et al. 1994; Lees 1993). In this context, oxidative stress has also been an important mechanism of damage caused by Hcy (Streck et al. 2003; Wyse et al. 2002). Studies demonstrated that Hcy chronic administration increased lipid peroxidation, reduced antioxidant defenses and total thiol content in the liver and blood of the rats (Matté et al. 2009a,b).

Vitamin C is a hydrosoluble vitamin widely present in many biological systems and in multivitamin preparations and it is commonly used to supplement inadequate dietary intake and as an antioxidant (Fox et al. 1989). Although most mammals synthesize vitamin C via the hexuronic acid pathway in the liver (Banhegyi et al. 1997), humans must acquire the water-soluble micronutrient from

dietary sources. Besides its antioxidant activity, vitamin C is involved in a number of cellular functions such as collagen biosynthesis and iron transport (Kim et al. 2006), and is also a naturally occurring free radical scavenger (Knight et al. 1993). It has been reported that gluthatione reductase activity is affected by reactive oxygen species (ROS) and that vitamin c is capable of preventing this effect (El-Missiry 1999).

Based on above information our objectives in this study were to analyze the effect of Hcy on glutamate uptake, Na^+,K^+- ATPase activity, and on oxidative stress parameters in hippocampus of young rats and also evaluate the protective effect of vitamin C on the damage to these parameters.

MATERIALS AND METHODS

Animals and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant room temperature (22 ± 1°C). The rats had free access to water and commercial protein chow. Animal care followed the NIH ‘‘Guide for the Care and Use of Laboratory Animals’’ (NIH publication no. 80-23, revised 1996) and our study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul.

L-[2,3-3H] glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK. The other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Chronic homocysteine and vitamin C administration

D, L- Hcy was dissolved in saline solution (0,85% NaCl) and buffered to pH 7.4. Hcy solution (0.3-0.6 µmol/g body weight) was administered subcutaneously twice a day at 8 h interval from their 6^{th} to their 21^{st} days of age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al. 2002). Vitamin C solution (100 mg/kg body weight) was injected intraperitoneally, once a day, also from 6^{th} to 21^{st} day of life. Control animals received saline solution in the same volumes as those applied to Hcy and vitamin C treated rats. The rats were sacrificed by decapitation twelve hours after the last injection and the brain was carefully removed and hippocampus was dissected.

Tissue and homogenate preparation

In order to measure the Na^+,K^+-ATPase activity, the hippocampus was homogenized in 10 volumes of 0.32mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 3000 x RPM for 10 min at 4 °C. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

To determine the oxidative stress parameters, the hippocampus was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 3000 x RPM for 10 min at 4 °C, to discard the nuclei and the cell debris. The pellet was discarded and the supernatant was taken to biochemical assays. The homogenates used were from individual animals, and they were never pooled. All experiments were repeated with different animals.

Glutamate uptake assay

Glutamate uptake was performed according to Frizzo et al. (2002). Hippocampus was cut into 400 µm thick slices with a McIlwain chopper. Briefly, slices were pre-incubated in Hank's balanced salt solution (HBSS) at 35°C for 15 min, followed by adding a solution containing 0.33 mCi/mL L-[2,3-3 H] glutamate with 100 µM unlabeled glutamate at 35 °C. Incubation was stopped after 5 min with two ice-cold washes of 1 ml HBSS, immediately followed by the addition of 0.5 N NaOH. Sodium-independent uptake was determined on ice (4°C), using N- methyl- D- glucamine instead of sodium chloride. Both the specific and non-specific uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter. Protein content was measured by Peterson method (Peterson 1977).

Na^+,K^+-ATPase activity assay

For determination of Na^+,K^+-ATPase activity, the hippocampus was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 170 µL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described (Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific

activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

Catalase assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The method used is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein.

Superoxide Dismutase Assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is substrate for SOD . The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double beam spectrophotometer with temperature control (Marklund, 1985). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. A 50% inhibition of pyrogallol autoxidation is defined as 1 unit of SOD and the specific activity is represented as units per mg protein.

2′7′dichlorofluorscein oxidation assay

Reactive species production was measured following Lebel et al. (1992) method based on 2′7′-dichlorofluorescein (H_2DCF) oxidation. Samples (60 μL) were incubated for 30 min at 37 °C in the dark with 240 μL of 100 μM 2′7′-dichlorofluorscein diacetate (H_2DCF-DA) solution in a 96 wells plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by reactive species presenting in samples. The last reaction produces the fluorescent compound DCF which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/ mg protein.

Protein determination

The protein content of hippocampus samples was determined using bovine serum albumin as standard, according to Lowry et al. (1951) or Bradford (1976).

Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan`s 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. Differences were considered statistically significant if $p<0.05$.

RESULTS

Initially, we investigated the effect of chronic administration of Hcy on glutamate uptake. Fig. 1. shows that chronic hyperhomocysteinemia significantly decreased glutamate uptake in hippocampus of young rats when compared to controls (saline administration). Besides, vitamin C treatment is ineffective, *per se*, but prevented the reduction in glutamate uptake caused by chronic Hcy administration [$F(3,20) = 4,291$; $p < 0.05$].

Next, we evaluated the effect of chronic hyperhomocysteinemia on Na^+,K^+-ATPase activity. Fig. 2 shows that rats subjected to hyperhomocysteinemia present a significant reduction of Na^+,K^+-ATPase activity in homogenate of hippocampus of young rats. In addition, this figure also shows that vitamin C, *per se*, did not alter the Na^+,K^+-ATPase activity, but when administered concomitantly with Hcy, it was able to prevent the damage caused by Hcy [$F(3,20) = 4,122$; $p < 0.05$].

We also investigated some parameters of oxidative stress in order to determine the involvement of reactive species in the hippocampus of young rats subjected to hyperhomocysteinemia. Fig. 3 shows that Hcy increased the levels of reactive species in this cerebral structure. Moreover, vitamin C, *per se*, significantly increased the amount of reactive species, but when administered concomitantly with Hcy, the vitamin was able to prevent the damage caused by the aminoacid [$F(3,24)= 4,34$; $p < 0.01$].

Finally, the effect of chronic hyperhomocysteinemia on antioxidant enzymes CAT and SOD in hippocampus of young rats was also evaluated. Fig. 4 shows that Hcy significantly reduced the activities of CAT [$F(3,24) = 3,759$; $p < 0.05$] and SOD [$F(3,24) = 8,324$; $p < 0.001$]. Furthermore, vitamin C, *per se*, did not alter the SOD and CAT activities, but prevented the inhibition of these enzymes caused by Hcy.

DISCUSSION

Inborn errors of metabolism (IEM) are multifactorial diseases, mostly autosomal recessive, covering genetic and biochemical changes. These disorders involve chemical changes in phenotype as a

consequence of blocking the metabolic pathways where these anomalous enzymes are inserted (Scriver et al. 2001). Homocystinuria is an IEM, which occurs due to the accumulation of Hcy, a methionine-derived sulphur amino acid. Clinically, affected patients present neurological manifestations such as mental retardation, psychiatric disturbances, and seizures (Mudd et al. 2001), whose the physiopatology is still unknown.

Studies performed in our laboratory indicate that hyperhomocysteinemia decreases the glutamate uptake and the Na^+,K^+-ATPase activity in brain of rats. On this basis, we decided to extend our investigation to elucidate the possible mechanisms involved in such alterations. Furthermore, the influence of vitamin C on the effects elicited by Hcy was also investigated. Besides, in order to clarify the involvement of oxidative stress in the effects elicited by Hcy, parameters such as production of reactive species (DCFH) and antioxidant enzymes CAT and SOD were evaluated. We used hippocampus because this cerebral structure is involved whith memory and is known that Hcy cause memory deficit in rats in the Morris water maze task (Streck et al. 2004). Our results showed that Hcy chronic administration decreased glutamate uptake in hippocampus of young rats. Moreover, data showed that vitamin C significantly prevented this effect. In fact, there are several evidences that glutamate transport can be impaired by free radicals, by direct oxidation of SH-group of the carrier protein (Sheldon et al. 2007; Volterra et al. 1994). In this context, Hcy has been shown as a potent oxidizing agent of SH-group by induce reactive species production, such as peroxynitrite (Radi et al. 1991), which are produced by Hcy autooxidation (Dayal et al. 2004; Faraci and Lentz 2004).

Na^+,K^+-ATPase is involved in several physiological functions such as regulation of the cell volume, cell differentiation, and maintenance of sodium and potassium equilibrium through biological membranes (Erecinska and Silver 1994). Therefore, another possible mechanism for glutamate uptake dysfunction is the inhibition of Na^+,K^+-ATPase, resulting in loss of the Na+ concentration gradient (Danbolt 2001). In this work, we demonstrated that chronic hyperhomocysteinemia decreased the Na^+, K^+- ATPase activity in hippocampus of young rats. Considering that, GLAST and GLT-1, which are dependent of sodium-gradient, could reverse the direction of glutamate transport, resulting in accumulation of extracellular glutamate (Maragakis et al. 2004).

Since previous studies showed that acute hyperhomocysteinemia decreased tissue antioxidant potential in the brain of the rats (Wyse et al. 2002) and considering that oxidation of Hcy may leads to the

formation of superoxide and hydrogen peroxide which subsequently alter neuronal function and predispose the affected neuronal tissue to neurodegenerative diseases (White et al. 2001), here we also investigated some oxidative stress parameters in order to elucidate these possible mechanism which could be involved in Hcy neurotoxicity. Results showed that chronic administration of Hcy enhanced the oxidation of DCFH in hippocampus of young rats, and that antioxidant vitamin C was able to prevent such effect. However, we observed that vitamin C, *per se*, also increased the DCFH oxidation. This finding is in agreement as it has been demonstrated by Brömme et al. (2008). In this sense, recent studies also have shown that vitamin C may act as prooxidant generating hydrogen peroxide and ascorbate radical at pharmacologic concentrations (Chen et al. 2007; Chen et al. 2008).

Regarding the enzymatic antioxidant defense that are responsible for removing ROS, our results showed that chronic administration of Hcy reduces CAT and SOD activities. Furthermore, vitamin C administration prevented the reduction of antioxidant enzymes in hippocampus of young rats. We believe that this imbalance between antioxidant enzymes caused by Hcy probably alter reactives species elimination, increasing the free radicals amount, in special ROS. These effects of Hcy could be responsible for, at least in part, the ability of Hcy to inhibit the expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (Upchurch et al. 1997; Yamamato et al. 2000). In addition, it is known that free radicals can decrease the Na^+,K^+-ATPase activity (Yufu et al. 1993) and it could be one of the mechanisms which Hcy acts.

In conclusion, we showed that Hcy chronic administration decreases glutamate uptake, Na^+,K^+-ATPase activity, enzymatic antioxidant defenses and enhanced reactive species, characterizing this amino acid as an oxidative stressor. Concomitantly, we showed that vitamin C administration prevented Hcy effects, possibly by its antioxidant features. Considering that oxidative stress elicited by Hcy could contribute to pathophysiology of disorders that accumulate this amino acid such as metabolic and neurodegenerative disorders, and if confirmed in human beings, we might propose the use of vitamin C as an adjuvant therapy in hyperhomocysteinemic patients. However, we reinforce the importance of careful prescription of vitamin C for healthy people, because of their possible prooxidant properties.

Acknowledgements

This work was supported in by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) and by the FINEP Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net) - # 01.06.0842-00".

REFERENCES

Aebi H (1984) Catalase in vitro. Methods Enzymol 105: 121-126.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-die-binding. Anal Biochem 72: 248-254.

Banhegyi G, Braun L, Csala M, Puskas F, Mandl J (1997) Ascorbate metabolism and its regulation in animals. Free Radic Biol Med 23: 793-803.

Brömme H-J , Zühlke L , Silber R-H , Simm A (2008) DCFH2 interactions with hydroxyl radicals and other oxidants – Influence of organic solvents. Experimental Gerontology 43: 638-644.

Chan KM, Delfert D et al (1986) A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal Biochem 157(2): 375-80.

Chen Q, Espey MG, Sun AY, Lee JH, Krishna MC, Shacter E et al (2007) Ascorbate inpharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. Proc Natl Acad Sci USA 104: 8749-54.

Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC et al (2008) Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci USA 105: 11105-11109.

Collingridge GL, Lester RAJ (1989) Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev 40: 143-210.

Dayal S, Arning E, Bottiglieri T, Boger RH, Sigmund CD, Faraci FM, Lentz SR (2004) Cerebral vascular dysfunction mediated by superoxide in hyperhomocysteinemic mice. Stroke 35: 1957-1962.

Danbolt NC (2001) Glutamate uptake. Progress in Neurobiology 65: 1-105.

El-Missiry MA (1999) Enhanced testicular antioxidant system by ascorbic acid in alloxan diabetic rats. Comparative Biochemistry and Physiology. Part C. Pharmacology, Toxicology & Endocrinology 124: 233-237.

Erecinska M, Silver IA (1994) Ions and energy in mammalian brain. Prog Neurobiol 43: 37-71.

Faraci FM, Lentz SR (2004) Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. Stroke 35: 345-347.

Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42: 1-11.

Fox BA and Cameron AG (1989) Food Science. In: Arnold E (ed) Nutrition and Health, London.

Frizzo ME, Lara DR et al (2002) Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell Mol Neurobiol 22(3): 353-63.

Gallucci M, Zanardo A, De Valentin L, Vianello A (2004) Homocysteine in Alzheimer disease and vascular dementia. Arch Gerontol Geriatr Suppl (9): 195-200.

Headley PM and Grillner S (1990) Excitatory amino acids and synaptic transmission: the evidence for a physiological function. Trends Pharmacol Sci 11: 205-211.

Herrmann W, Lorenzl S, Obeid R (2007) Review of the role of hyperhomocysteinemia and B-vitamin deficiency in neurological and psychiatric disorders- current evidence and preliminary recommendations. Fortschr Neurol psychiatr 75: 515-527.

Hitschke K, Buhler R, Apell HJ and Stark G (1994) Inactivation of the Na^{+},K^{+-}ATPase by radiation-induced free radicals. Evidence for a radical-chain mechanism. FEBS Lett 353: 297-300.

Kim H, Lee S, Lee D, Smith D, Jo H, Schellhorn H and Boo Y (2006) Biochem Biophys Res Commun 345: 1657-1662.

Kuhn W, Roebroek R, Blom H, Van Oppenraaj D, Przuntek H, Kretschmer A, Buttner T, Woitalla D, Muller T (1998) Elevated plasma levels of homocysteine in Parkinson's disease. Eur Neurol 40: 225-227.

Lebel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5: 227-231.

Lees GJ (1993) Contributory mechanisms in the causation of neurodegenerative disorders. Neuroscience 54: 287-322.

Lipton SA, Kim WK, Choi YB, Kumar S, D'Emilia DM, Rayudu PV, Arnelle DR, Stamler JS (1997) Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 94: 5923–5928.

Lowry OH, Rosebrough NJ, Farr AL, RandalL RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-267.

Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 15: 461-473.

Marklund SL (1985) Pyrogallol autoxidation. In: Handbook for Oxygen Radical Research, CRC Press, Boca Raton, pp. 243–247.

Matté C, Mackedanz V, Stefanello FM, Scherer EB, Andreazza AC, Zanotto C, Moro AM, Garcia SC, Gonçalves CA, Erdtmann B, Salvador M, Wyse AT (2009) Chronic hyperhomocysteinemia alters antioxidant defenses and increases DNA damage in brain and blood of rats: protective effect of folic acid. Neurochem Int 54(1): 7-13.

Matté C, Stefanello FM, Mackedanz V, Pederzolli CD, Lamers ML, Dutra-Filho CS, Dos Santos MF, Wyse AT (2009a) Homocysteine induces oxidative stress, inflammatory infiltration, fibrosis and reduces glycogen/glycoprotein content in liver of rats.Int J Dev Neurosci 27(4): 337-344.

Matté C, MussulinI BH, Dos Santos TM, Soares FM, Simão F, Matté A, De Oliveira DL, Salbego CG, Wofchuk ST, Wyse AT (2010) Hyperhomocysteinemia reduces glutamate uptake in parietal cortex of rats. Int J Dev Neurosci 28(2): 183-187.

Mattson MP, Chan SL, Duan W (2002) Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. Physiological Reviews 82: 637-672.

Mudd SH, Levy HL, Skovby F (2001) Disorders of transsulfuration. In: Scriver, CR, Beaudet AL, Sly WS, Valle D (Eds) The Metabolic and Molecular Basis of Inherited Disease, vol. 2, McGraw-Hill, New York, pp. 1279–1327.

Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83(2): 346-56.

Sachdev PS (2004) Homocysteine and neuropsychiatric disorders. Rev Bras Psiquiatr 26: 50-56.

Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. J Biol Chem 266: 4244-4250.

Schalinske KL (2009) Hepatic sulfur amino acid metabolism. In: Masella R and Mazza G (eds) In glutathione and Sulfur Amino Acids in Human Health and Disease, Wiley, Hoboken, New Jersey, pp. 73-90.

Scriver CR, Beaudet AL, Sly WS, Valle D (2001) The metabolic & molecular bases of inherited disease. McGraw-Hill, New York.

Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51: 333-355.

Sjöström M, Stenström K, Eneling K, Zwiller J, Katz AI, Takemori H, and Bertorello AM (2007) SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process. Proc Natl Acad Sci USA 104: 16922-16927.

Streck EL, Vieira PS, Wannmacher CM, Dutra-Filho CS, Wajner M, Wyse AT (2003) In vitro effect of homocysteine on some parameters of oxidative stress in rat hippocampus. Metab Brain Dis 18(2): 147-154.

Streck EL, Matte C et al (2002) Reduction of Na^+,K^+-ATPase activity in hippocampus of rats subjected to chemically induced hyperhomocysteinemia. Neurochem Res 27(12): 1593-1598.

Streck EL, Bavaresco CS, Netto CA, Wyse AT (2004) Chronic hyperhomocysteinemia provokes a memory deficit in rats in the Morris water maze task. Behavioural Brain Research 153: 377-381.

Upchurch GR, Jr Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF Jr and Loscalzo J (1997) Homocysteine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 272: 17012-17017.

Volterra A, Trotti D, Racagni G (1994) Glutamate uptake is inhibited by arachidonic acid and oxygen radicals via two distinct and additive mechanisms. Mol Pharmacol 46: 986-992.

White AR, Huang X, Jobling MF, Barrow CJ, Beyreuther K, Masters CL, Bush AL, Cappai R (2001) Homocysteine potentiates copper- and amyloid beta peptide-mediated toxicity in primary neuronal cultures possible risk factors in the Alzheimer's-type neurodegenerative pathways. J Neurochem 76: 1509-1520.

Wyse AT, Streck EL et al (2000) Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem Res 25(7): 971-975.

Wyse AT, Zugno AI, Streck EL, Matté C, Calcagnotto T, Wannmacher CMD, Wajner M (2002) 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 27: 1685-1689.

Yamamato M, Hara H and Adachi T (2000) Effects of homocysteine on the binding of extracellular-superoxide dismutase to the endothelial cell surface. FEBS Lett 486: 159-162.

Yufu K, Itoh T, Edamatsu R, Mori A and Hirakawa M (1993) Effect of hyperbaric oxygenation on the Na^+,K^+-ATPase and membrane fluidity of cerebrocortical membranes after experimental subarachnoid hemorrhage. Neurochem Res 16: 1033-1039.

Zieminska E, Lazarewicz JW (2006) Excitotoxic neuronal injury in chronic homocysteine neurotoxicity studied in vitro: the role of NMDA and group I metabotropic glutamate receptors. Acta Neurobiol Exp (Wars) 66: 301-309.

Legends to figures

Fig. 1 Effect of chronic administration of homocysteine, vitamin C and homocysteine plus vitamin C on glutamate uptake in hippocampus of young rats. Data are mean ± S.D. for 5-6 animals in each group. Results are expressed in nmol /min/ mg protein. * $P<0.05$ compared to control (Duncan multiple range test). Hcy, homocysteine; Vit, vitamin.

Fig. 2 Effect of chronic administration of homocysteine, vitamin C and homocysteine plus vitamin C on Na^+,K^+-ATPase activity in homogenates from hippocampus of young rats. Data are mean ± S.D. for 5-6 animals in each group. Results are expressed in nmol Pi/ min mg protein. * $P<0.05$ compared to control (Duncan multiple range test). Hcy, homocysteine; Vit, vitamin.

Fig. 3 Effect of chronic administration of homocysteine, vitamin C and homocysteine plus vitamin C on the levels of reactive species in hippocampus of young rats. Data are mean ± S.D. for 5-6 animals in each group. Results are expressed in nmol/ mg protein. ** $P<0.01$ compared to control (Duncan multiple range test). Hcy, homocysteine; Vit, vitamin.

Fig. 4 Effect of chronic administration of homocysteine, vitamin C and homocysteine plus vitamin C on antioxidant enzyme activities CAT (A) and SOD (B) in hippocampus of young rats. Data are mean ± S.D. for 5-6 animals in each group. Results are expressed in U/ mg protein. * $P<0.05$ compared to control; *** $P<0.001$ compared to control (Duncan multiple range test). CAT, catalase; SOD, superoxide dismutase; Hcy, homocysteine; Vit, vitamin.

Fig. 1

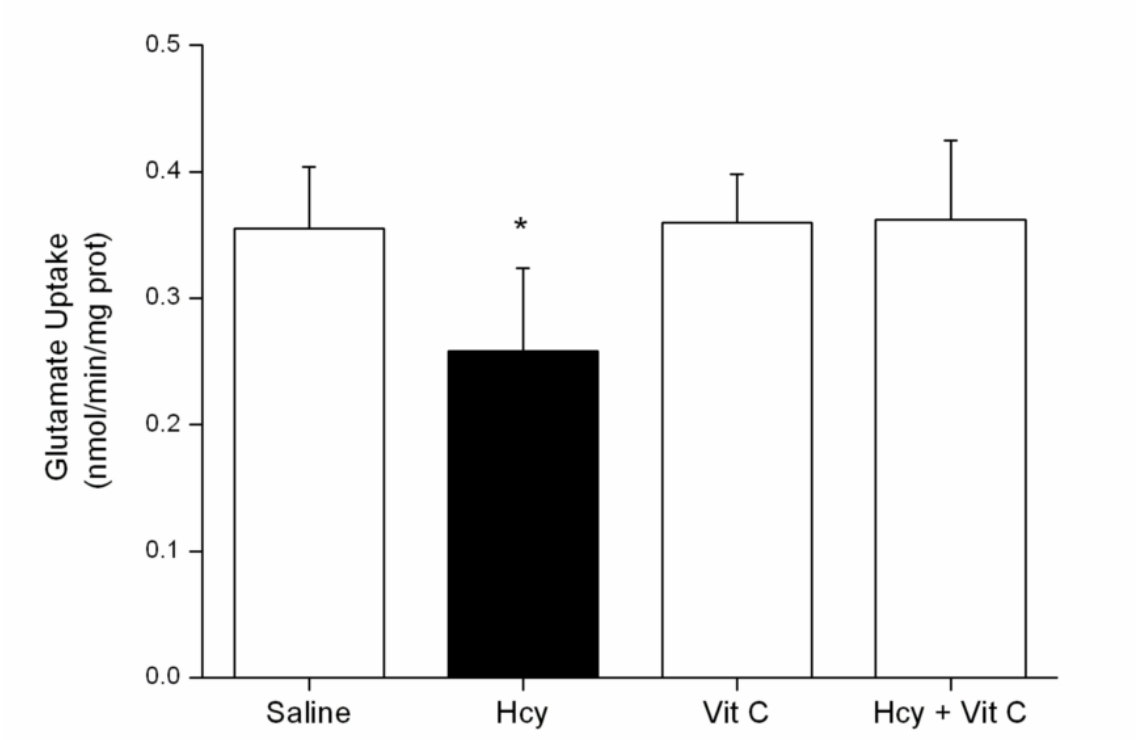

Glutamate Uptake
(nmol/min/mg prot)
0.5
0.4
0.3
0.2
0.1
0.0
*
Saline
Hcy
Vit C
Hcy + Vit C

Fig. 2

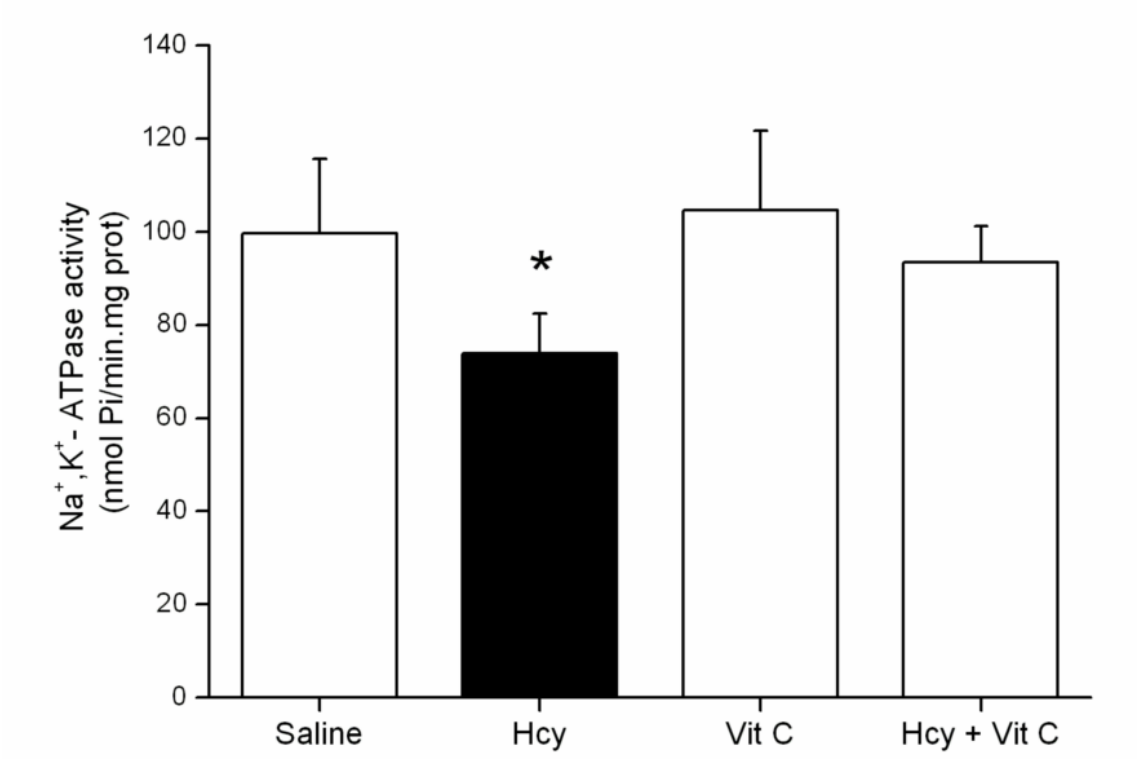

140
120
100
80
60
40
20
0
Na+,K+- ATPase activity
(nmol Pi/min.mg prot)
*
Saline
Hcy
Vit C
Hcy + Vit C

Fig. 3

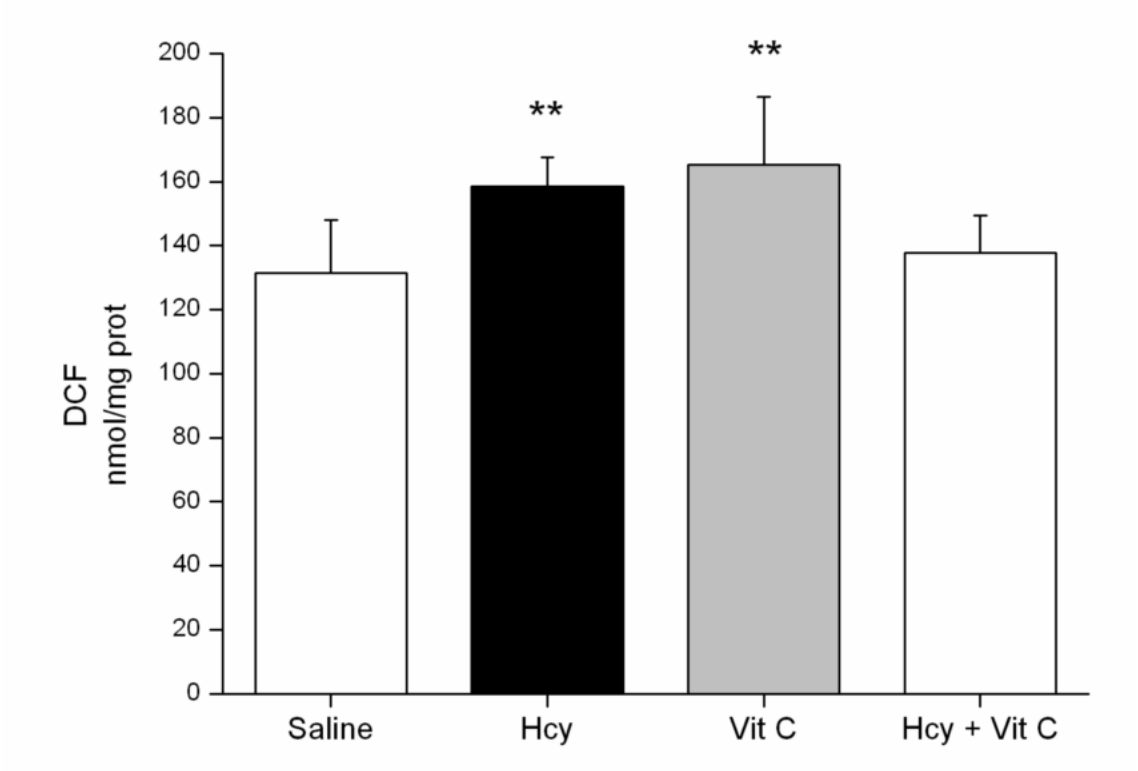
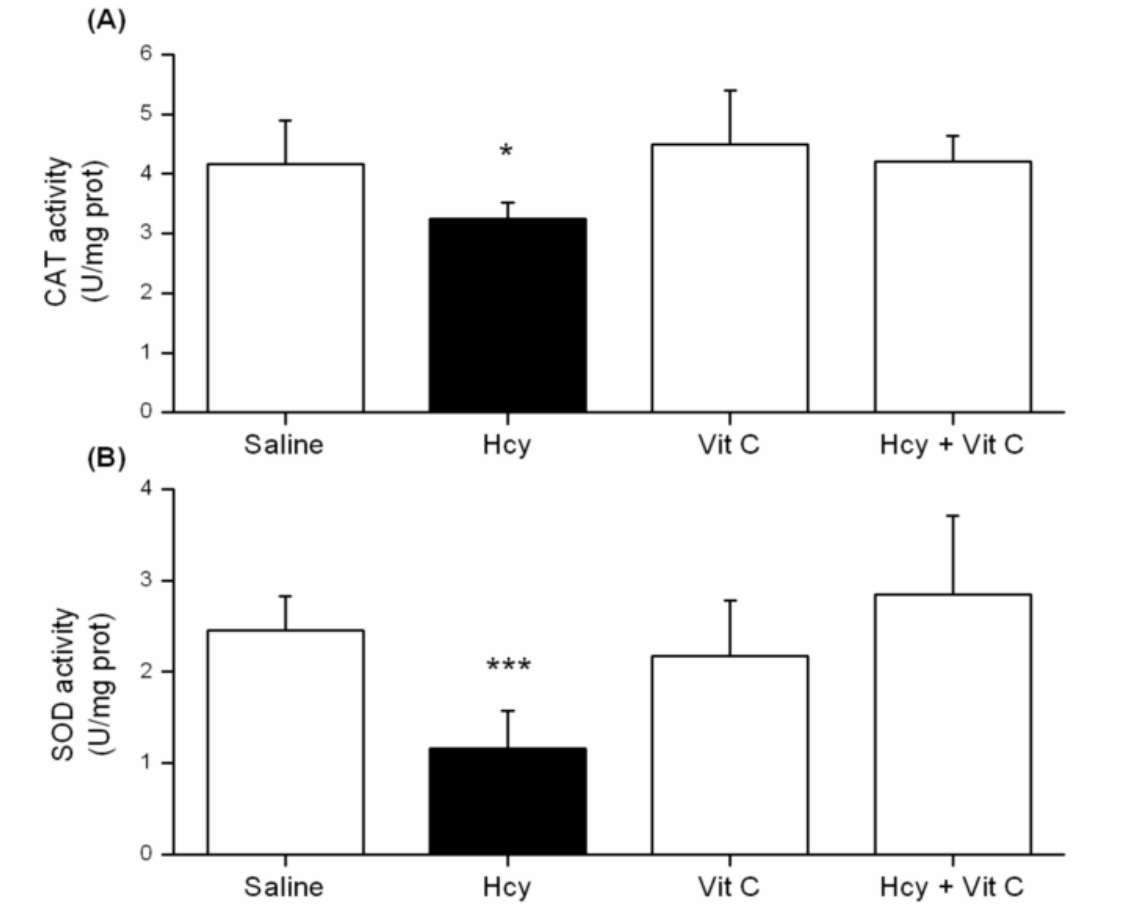

DCF
nmol/mg prot
200
180
160
140
120
100
80
60
40
20
0
**
**
Saline
Hcy
Vit C
Hcy + Vit C

Fig. 4


(A)
CAT activity (U/mg prot)
6
5
4
3
2
1
0
*
Saline
Hcy
Vit C
Hcy + Vit C
(B)
SOD activity (U/mg prot)
4
3
2
1
0
***
Saline
Hcy
Vit C
Hcy + Vit C

Anexo 1

JOURNAL OF NEURAL TRANSMISSION- INSTRUCTIONS FOR AUTHORS

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please follow the hyperlink "Submit online" on the right and upload all of your manuscript files following the instructions given on the screen.

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.

Note: If you use Word 2007, do not create the equations with the default equation editor but use the Microsoft equation editor or MathType instead.

- Save your file in doc format. Do not submit docx files.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1993).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations.