



NutriSim© ensures against hippocampal neuronal harm incited by ischemia-reperfusion in Mongolian gerbils (*Meriones unguiculatus*): Morphological and biochemical methodology

Velázquez Brizuela, J. A Cruz-Ramos and Genaro Gabriel

¹Laboratorios BioSim©; Área de Desarrollo e Investigación Científica, México.

Abstract

Mongolian gerbil model has been extensively used for the study of neuroprotective drugs since transient bilateral common carotid artery occlusion induces neuronal cell death to selectively vulnerable regions, including the CA1 sector of the hippocampus. Oxidative stress is strongly involved in this phenomenon. NutriSim© a nutritive supplement has been used empirically in the treatment of several degenerative disorders. Therefore in the present work we studied the ability of NutriSim© to protect against brain damage induced by ischemia-reperfusion in Mongolian gerbils. Brain damage was monitored by histological analysis of CA1 region and quantitative determinations of lipoperoxides, nitric oxide catabolites (nitrates/nitrites) and Glutathione peroxidase enzyme (GPx) activity. We found that a single dose of NutriSim© reduces hippocampal neuronal death in the CA1 of the hippocampus and attenuates increases in lipid peroxidation products (malondialdehyde and 4- hydroxyalkenals), nitric oxide catabolites (nitrates/nitrites) and GPx activity. These results suggest that neuroprotective effects of NutriSim© are partly attributed to its antioxidant action.

Keywords: NutriSim©, hippocampus, ischemia-reperfusion.

INTRODUCTION

Cerebrovascular diseases, including stroke, are associated with high mortality worldwide and ischemic stroke accounts for about 88 percent of all strokes. The most important etiologies of ischemic stroke include large-artery atherosclerosis, cardioembolism and cerebral small-vessel disease (Rosamond et al., 2008).

The brain represents a structure extremely sensitive to hypoxia and the Mongolian gerbil (*Meriones unguiculatus*) has

been widely used as a model for investigating the cerebral consequences of ischemia because of its unusual cerebral circulation which lacks connections between the carotid and vertebralbasilar circulations, therefore there is an incomplete arterial circle and the temporary bilateral occlusion of common carotid arteries provides a simple and reproducible approach to transient brain ischemia. A transient carotid occlusion for 5 min induces delayed neuronal death in the hippocampal

CA1 region (Kirino, 1982). This experimental model represents human stroke conditions due, for example, to atherosclerotic involvement of the common carotid arteries, respiratory arrest and cardiac arrest (Adams et al., 1993).

Reactive oxygen species (ROS) and nitric oxide (NO) also participate in the pathogenesis of cerebral injury induced by ischemia and reperfusion and oxygen free radicals are elevated during cerebral ischemia and reperfusion because of the failure of metabolic reactions (Fujimura et al., 2005; Moro et al., 2005). Activation of nitric oxide synthase leads to excess production of NO. High concentrations of NO are toxic and interact with superoxide radical (O_2^-) to produce the highly toxic peroxynitrite anion ($ONOO^-$) (Traystman, 1991). These radicals react with intracellular proteins, lipids, sugars and nucleotides and impair the normal function and physiology of cells. Antioxidant defences including free radical scavengers and antioxidative enzymes are considered as the most effective means at attenuating the tissue injury induced by ischemia and reperfusion (Papadopoulos et al., 1998).

NutriSim® is a nutritional supplement that contains arginine, lysine, choline, ammonium chloride, calcium and magnesium. It is used empirically for several degenerative diseases. Using the gerbil model, it has been found that a single dose of NutriSim®, 30 min prior the ischemic treatment reduces significantly the brain edema (Miranda-Riestra, 2004).

Therefore to elucidate its possible mechanism of action we investigated the effect of NutriSim® on hippocampal delayed neuronal death and some markers of oxidative stress after transient cerebral ischemia.

MATERIALS AND METHODS

Animal model

180 adult male Mongolian gerbils weighing 90 - 100 g were maintained on a 12 h/ 12 h light/dark cycle at 22°C and given access to food (Chow Purina) and water *ad libitum*. All animal experiments were approved by the ethical committee (Mexico) and were conformed to international guidelines on the ethical use of animals. Animals were randomly assigned into two groups: I) 120 animals for the biochemical determinations and II) 60 animals for the morphological evaluation. These groups in turn were subdivided evenly into five subgroups: subgroup 1, for control non-treated; subgroup 2, for sham operated; subgroup 3, underwent the surgical procedure of ischemia and reperfusion; subgroup 4, was intraperitoneally (i.p.) injected with a single dose of NutriSim® (10 l/100 g of body weight) 30 minutes before ischemia; subgroup 5, was a non-ischemic group receiving a single dose of NutriSim® (10 l/100 g of body weight).

Experimental procedures

Animals were anesthetized with droperidol (2.5 mg/Kg subcutaneous) and ketamine (50 mg/kg intramuscular). Bilateral common carotid arteries were dissected and occluded with atraumatic microaneurism clips (Roboz Surgical Instruments, Rockville, MD).

Occlusion was induced for 5 min to produce transient global ischemia of the forebrain. In sham-operated animals, both common carotids were exposed but not occluded. After removal of the clips, restoration of blood flow was verified by direct observation through a microscope. The surgical incision was sutured and disinfected and animals were returned to their cages. The body temperature ($37 \pm 0.5^\circ\text{C}$) was maintained with a heating blanket before, during the surgery and after the surgery until the animals fully recovered from anaesthesia.

Eight animals were killed at 90 min, 120 min and 7 days following the reperfusion insult. After decapitation, hippocampi were dissected and homogenized separately in ice-cold 20 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4), 0.25 M sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol with a polytron like stirrer. Homogenates were centrifuged at 15,000 g for 15 min at 4°C and the supernatant was collected and immediately assayed for biochemical determinations. Biochemical and histological data were obtained by two independent investigators who were unaware of the treatment protocol.

Histological analysis

Sixty animals were re-anesthetized using the same protocol as previously described and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 90 min, 120 min and 7 days after ischemia-reperfusion treatment.

Brains were removed and postfixed in the same fixative for more than 12 hours, processed and embedded in paraffin wax. Then, 5 m thick paraffin cross sections containing hippocampal tissue were mounted on slides and stained with haematoxylin and eosin. Microscopy was conducted on a Zeiss Axioscop (Goettingen, Germany) and images were captured and processed by a Spot CCD camera.

Lipid peroxidation

Lipid peroxidation products were assayed using a Kit from Oxford Biomedical Research Incorporated. (product No. FR 12). The Kit contains a chromogenic reagent (N-methyl-2-phenylindole) which reacts with the lipid peroxidation products malonaldehyde (MDA) and 4-Hydroxyalkenals (4-OHA) at 45°C yielding a stable chromophore with maximal absorbance at a wavelength of 586 nm (Esterbauer and Cheeseman, 1990). Results are expressed as $\text{nmol MDA}+4\text{-OHA (mg protein)}^{-1}$.

Nitric oxide catabolites (nitrates/nitrites)

Nitric oxide release was determined spectrophotometrically by measuring the accumulation of its stable degradation products, nitrite and nitrate. Quantification of these metabolites was done using a commercial package (Calbiochem Nitric Oxide Assay Kit, colorimetric 482650). Briefly, nitrate to nitrite conversion was done using nitrate reductase. Total nitrite is then determined spectrophotometrically by using the Griess reaction (Griess, 1864).

Determination of glutathione peroxidase activity

The protocol used was a modification of the method described previously (Jaskot et al., 1983). Briefly, homogenized hippocampi were centrifuged at 15,000 $\times g$ for 15 min at 4°C and the supernatant was decanted and re-centrifuged at 100 000 $\times g$ for 60 min at 4°C. This supernatant was diluted 1:2 times with 50 $\mu\text{mol/L}$ of potassium chloride (pH 7.6). Glutathione peroxidase (GPx) was measured by a coupled reaction with glutathione reductase (GRd).

using cumene hydroperoxide as substrate and measuring the decrease in the NADPH absorbance at 340 nm. The product of GPx, GSSG, acts as a substrate for GRd. The primary role of GRd is to replenish the levels of GSH that have been oxidized by free radicals. The GRd activity was measured by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP in the presence of GSSG. This activity was referred to as micromoles of oxidized NADPH/min/mg of protein (oxidized NADPH μ M min/mg protein).

Determination of Protein

Proteins were determined by the microassay procedure using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules CA).

Statistical evaluation

All values reported are expressed as mean \pm standard error. Statistical significance of differences among groups was tested by one-way analysis of variance (ANOVA), followed by multiple comparisons between each group and control or other groups using Dunnett's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Histological analysis

Normal structure of hippocampal pyramidal neurons are shown in Figures 1a and b (for control non-treated and non-ischemic group receiving a single dose of NutriSim $\text{\textcircled{C}}$, respectively). Macroscopically, at 90 and 120 min after ischemia the brains showed edema and widespread congestion, including NutriSim $\text{\textcircled{C}}$ treated groups. However, brain edema was reduced when NutriSim $\text{\textcircled{C}}$ was applied prior ischemia-reperfusion treatment. Sagittal section at the median eminence region was not altered macroscopically, in all groups. At day 7 after ischemia we did not observe any alteration both general and within the brain (data not shown).

Microscopic examination of CA1 region of hippocampus exhibited increased degenerative changes at different time after ischemia-reperfusion: hyperchromasia, widening of Virchow-Robin spaces, swelling of the nucleus and cellular shrinkage (Figures 1a - e). Furthermore, neuronphagia, astroglial aggregates and neuronal cell death were evident at 120 min and 7 days after ischemia-reperfusion (Figures 1d and e, respectively). Interestingly, we observed a protective effect of NutriSim $\text{\textcircled{C}}$, that is, hyperchromasia and astroglial aggregates were still evident, but minor compared with the ischemia-reperfusion control group (Figures 1f and g). In addition, delayed neuronal death, at 7 days of ischemia-reperfusion was reduced by NutriSim $\text{\textcircled{C}}$ (Figure 1h).

Biochemical analysis

To explore if the neuroprotective effect of NutriSim $\text{\textcircled{C}}$ was

Figure 1. Representative light micrographs of the CA1 sector region of dorsal hippocampus of Mongolian gerbils. Control without ischemia (1a), control with NutriSim $\text{\textcircled{C}}$ (1b). Ischemia-reperfusion groups, at 90 min (1c), 120 min (1d) and 7 days (1e). NutriSim $\text{\textcircled{C}}$ plus ischemia groups, at 90 min (1f), 120 min (1g) and 7 days (1h). (Curly bracket, CA1 region; arrow, neurons).

mediated by a possible antioxidant action, we measured oxidative stress markers in hippocampi homogenates. As shown in Figures 2 and 3, respectively a basal level of lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals) and nitric oxide catabolites (nitrates/nitrites) were detected in hippocampi from control, ischemia and NutriSim $\text{\textcircled{C}}$ treated gerbils. A minor increment in lipid peroxidation products (Figure 2) and nitric oxide catabolites (Figure 3) were detected in sham-operated animals, but there was a significant increase in lipid oxidation products in hippocampi isolated from gerbils subjected to the treatment of ischemia-reperfusion from 90 and 120 min. Interestingly, a single dose of NutriSim $\text{\textcircled{C}}$, 30 minutes before the ischemia treatment attenuate the increase in lipid oxidation products and nitric oxide catabolites, quantified to 90 and 120 min after reperfusion. At 7 days after treatment, there was not a statistically significant change between treatments, but

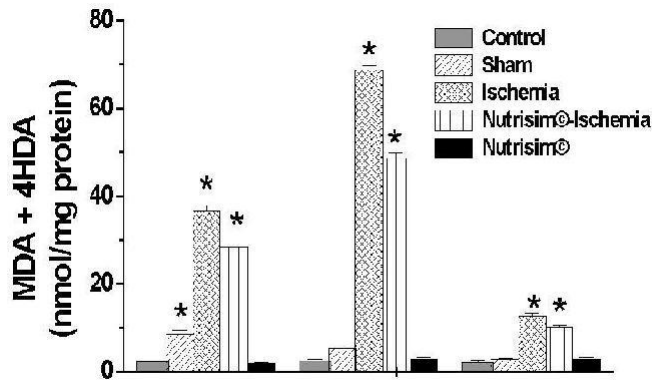


Figure 2. Lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals) in hippocampi from the indicated gerbils groups at 90 min, 120 min and 7 days after reperfusion. Asterisks indicate a significant difference from the ischemia-reperfusion group ($P < 0.05$). The bar indicates the mean \pm S.E.

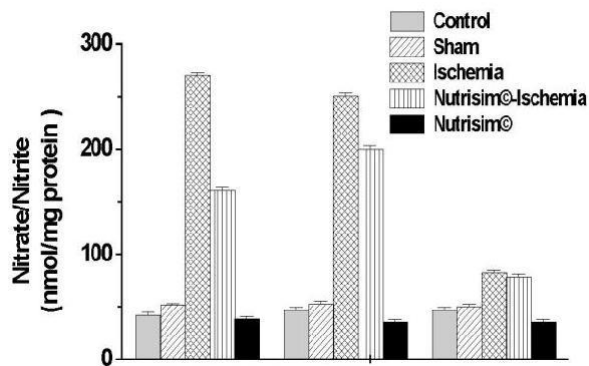


Figure 3. Nitric oxide catabolites in hippocampi from the indicated gerbils groups at 90 min, 120 min and 7 days after reperfusion. Asterisks indicate a significant difference from the ischemia-reperfusion group ($P < 0.05$). The bar indicates the mean \pm S.E.

the trend is similar to previous experiments.

Figure 4 shows that the enzyme GPx activity was increased significantly in hippocampi from gerbils subjected to ischemia and reperfusion with respect to the hippocampi from groups control, sham operated and NutriSim© (single dose). In contrast, Figure 4 also shows that a single dose of NutriSim© before ischemia prevents partially the increase in glutathione peroxidase activity. Interestingly, that enzymatic activity was diminished 7 days after treatment in the groups subjected to ischemia.

DISCUSSION

The present work demonstrates that intraperitoneal injection of NutriSim© 30 minutes prior to forebrain ischemia in gerbils is neuroprotective under the conditions of

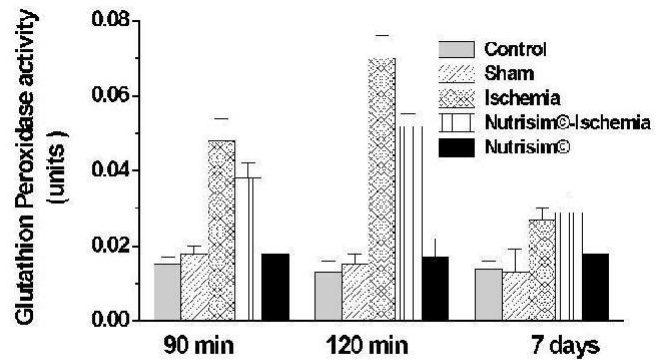


Figure 4. Glutathione peroxidase activity in hippocampi from the indicated gerbils groups at 90 min, 120 min and 7 days after reperfusion. Asterisks indicate a significant difference from the ischemia-reperfusion group ($P < 0.05$). The bar indicates the mean \pm S.E.

bilateral carotid occlusion assayed. These data implicated that NutriSim© is capable of passing the brain-blood barrier and exerting neuroprotective effect in the CA1 region of the hippocampus. The results demonstrated that NutriSim© inhibited the production of lipid peroxidation and nitric oxide catabolites and also diminishes the glutathione peroxidase activity. The net effect may be the reduction in oxidative stress. In addition NutriSim© reduces cortical neuron injury induced by ischemia-reperfusion.

NutriSim© contains arginine, lysine, choline, ammonium chloride, calcium and magnesium. Its components could exert some important functions as described below. L-arginine (L-arg), a semi-essential amino acid, depending on its metabolic pathway, becomes very essential in stress situations such as heatstroke, burns, sepsis, trauma and wound healing (Chatterjee, 2006; Tong et al., 2004). Additionally, it has been demonstrated that L-arg diminishes lipid peroxidation in patients with diabetes mellitus (Lubec et al., 1997) and has promising results during treatment of cardiovascular diseases including atherosclerosis, hypertension, angina pectoris, hyperlipidemia, and some kidney disorders, (Clarkson et al., 1996). Arginine affects cellular defense function, presumably by means of inducible nitric oxide synthetase (iNOS) mediated NO formation. These effects of arginine on cellular defense function led to its inclusion into current concepts of immune enhancing formulas to reduce the incidence of infectious morbidity and mortality in immune compromised patients). Arginine is used as a supplement in the treatment of arterial heart disease (Fisman et al., 1999; Wu and Meininger, 2000).

Lysine is an essential amino acid. This means that it must be obtained through the diet in adequate quantities to meet the body needs. Lysine is required in the body for the manufacture of carnitine, which is an amino acid used for the proper metabolism of fats. Lysine deficiency can interfere with carnitine synthesis and have adverse

impact upon fat metabolism to energy (Viviani et al., 1966).

Choline a precursor of brain phospholipids and the neurotransmitter acetylcholine is an essential nutrient (Sheard and Zeisel, 1996) and has a neuroprotective effect in situations of hypoxia and ischemia in animal models (De Bruin et al., 2003). Interestingly, cytidine 5' diphosphocholine, a choline precursor has a protective effect in patients with an acute cerebral infarction (Tazaki et al., 1988). On the other hand, calcium and magnesium are essential minerals that are needed for a broad variety of physiological functions, for instance an increased magnesium supply, however, might contribute to a risk reduction towards various diseases as for example coronary artery disease or osteoporosis (Vorman, 2003). At this time, it is not known if the whole mixture of NutriSim® or a specific component is the responsible for its effects. However the effects found in this work warrants further investigation.

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