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Beneficial Effect of Nutrisim[®] against LPS-Induced Liver Injury: Oxidative Stress Markers and Mitochondrial ATP-ase Activity

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ABSTRACT

Sepsis is a systemic host response which implicates an inflammatory state due to a bacterial infection and involving multiple organ failure. In order to modulate this systemic reaction, liver plays a pivotal role due to its central contribution in metabolism and host defense mechanism. The process of sepsis as an inflammatory condition results in the production of free radicals, in some cases induced by lipopolysaccharide which conducts to oxidative damage and mitochondrial impairment; these alterations may contribute to the pathophysiology of septic shock. The use of antioxidants in the treatment of this pathology reduces mitochondrial damage and oxidative stress, therefore in this study, treatment with Nutrisim[®] a nutritive supplement used empirically in the treatment of several degenerative disorders was tested in biochemical parameters indicative of oxidative stress and mitochondrial ATPase activity in rat liver mitochondria damage-induced by LPS. The results showed that treatment with Nutrisim[®] 30 min prior or after the LPS challenge significantly attenuates the end-products of lipid oxidation ($p < 0.05$), restores total antioxidant capacity, the membrane fluidity and preserves ATPase activity. In conclusion, NutriSim[®] administration prior or after LPS damage, ameliorates liver damage. Therefore, the beneficial effects of Nutrisim[®] against oxidative stress and ATPase activity in rat liver mitochondria presented in this study may suggest a potential chemoprotective effect in sepsis prevention.

Key words: NutriSim[®], sepsis, mitochondria, oxidative stress, ATPase, membrane fluidity, lipoperoxidation

INTRODUCTION

Sepsis, a systemic host response to infection, with multiple organ dysfunction syndrome continues to be the main cause of morbidity and mortality in intensive care units. It is known that this syndrome generates acute body inflammation, with massive increases of nitric oxide (NO) levels and inflammatory factors like cytokines in biological fluids, leading to a systemic damage and impaired tissue (Pinsky, 2001). In the normal gastrointestinal tract, bacteria are a normal

constituent; however pathogenesis from Gram-Negative (GN) bacteria, can arise during systemic exposure to either enterobacteria or their constituents (Raetz, 1990). Recognition of bacterial components such as lipopolysaccharide (LPS) a major mediator of inflammation that results in an exaggerated pathogenic inflammatory response in the circulation and vital organs. LPS is likely responsible for many of the events that occur during sepsis (Rietschel *et al.*, 1994). Exposure to LPS produces an array of pathophysiological changes similar to those seen during sepsis, including hypotension and cytotoxic lesions as well as functional and metabolic disturbances in different organs (Deitch, 1992; Garcia *et al.*, 1990). The liver is one of the most important organs with regard to the production and effects of tumor necrosis factor- α (TNF α). First, liver Kupffer cells or macrophages are major sources of production of TNF α in the body in response to bacterial LPS (Karck *et al.*, 1988). Second, TNF- and other pro-inflammatory mediators, such as interleukins 1 (IL1) and 6 (IL6), are excessively released into circulation which may alter the liver functions, resulting in changes in the integrity of liver vasculature (Toledo-Pereyra and Suzuki, 1994) or the regulation of liver cytochrome P450 contents and metabolic capacity (Morgan, 1997). Furthermore, liver exerts an endotoxin clearance function in which both Kupffer cells and parenchymal cells participate (Portoles *et al.*, 1994).

Current evidence indicates that host mediators, such as TNF- α , stimulate the Reactive Oxygen (ROS) and Reactive Nitrogen (RNS) species production involved in bacterial infection. In addition, TNF- α production drives mitochondrial ROS generation; oxidants must play into inflammatory mitochondrial damage (Suliman *et al.*, 2004). Interestingly, it has been reported that LPS can lead to functional endoplasmic reticulum failure via a mitochondrion-dependent pathway in livers of rats (Kozlov *et al.*, 2009). This may be a reason for organ dysfunction upon excessive inflammatory response mediated by endotoxin. Furthermore, mitochondrial dysfunction in sepsis was related to an increase of ROS and RNS species (Lopez *et al.*, 2006).

The structure and function of cell membranes plays an important role in maintaining intracellular homeostasis through the activity of membrane enzymes, hormone receptors and the transmembrane transport system. These dynamic phenomena are influenced by membrane fluidity. Fluidity is an important characteristic of biologic membranes at a molecular level. It indicates the way and the rate of motion of molecules in the membrane and is inversely related to membrane microviscosity. Changes in membrane fluidity may play a role in the regulation of membrane properties, both under normal and pathological conditions (Schuller *et al.*, 1986; Kim *et al.*, 1988).

Mitochondria provide cellular energy via oxidative phosphorylation, using the multisubunit complexes of the respiratory chain to create a transmembrane potential which drives ATP synthesis by the ATP synthase; ATP synthase is also capable of working as an ATPase (Boyer, 1997).

NutriSim[®] (Biosim Laboratory, Mexico), a nutritive supplement used empirically in the treatment of several degenerative disorders, inhibits endotoxin-induced production of proinflammatory cytokines (Renovato *et al.*, 2012) and diminishes oxidative stress elicited by ischaemia-reperfusion (Davalos *et al.*, 2010). This nutritive supplement is a mixture of amino acids of low molecular weight that are found naturally in the human body: arginine, choline and lysine salts and minerals: magnesium chloride, calcium and ammonium (Romero-Davalos *et al.*, 2011).

In previous studies we have demonstrated that the administration of NutriSim[®] 15 min prior or after LPS challenge possibly enhances the immune system response (Romero-Davalos *et al.*, 2011), also the use of this supplement in toxicity studies (animal models), have demonstrated the safety of the use of this product; indicating safety and non-toxicity, no undesirable pharmacological

and side effects. The aim of this study was to examine the effects of NutriSim[®] on mitochondrial markers (oxidative stress, membrane fluidity and on the hydrolytic activity of ATP synthase) in rat liver in an experimental septic shock model.

MATERIALS AND METHODS

Animals: Total 50 adult male Wistar rats (obtained from the animal facility of Centro de Investigacion Biomedica de Occidente, Guadalajara, Jalisco, Mexico) weighing 200-250 g, maintained on a 12/12 h light-dark cycle at 22°C, fed with standard diet (Chow-Purina[®]) and water *ad libitum* were used for this research work. All animals received human care based on the international guidelines on the ethical use of animals and the “Norma Oficial Mexicana NOM-062-ZOO-1999”. Experimental procedures were approved by the local animal ethics committee (Guadalajara, Jalisco, Mexico).

Experimental design: Fifty animals were randomly assigned to five experimental groups (n = 10 per group); they underwent the treatment as presented below. The via of administration for all animals was intraperitoneally (i.p.), NutriSim[®] (provided by Biosim Laboratory, Mexico) established dose was (10 µL/100 g of body weight) (Davalos *et al.*, 2010) and for LPS (*Escherichia coli*, serotype 0111:B4, purchased from Sigma Chemical Co., St. Louis, MO.) using our established LD₁₀₀ (100% lethal) of (20 mg kg⁻¹ of b.wt.) (Renovato *et al.*, 2012); administration was carried out at the same time of the day. Each rat was used only for a single experiment:

Groups	Treatment
1- Control	Rats* were treated with a single dose of physiologic saline solution (0.9% of sodium chloride) administered by i.p. injection
2- Nutrisim [®]	Rats* were treated with a single dose of NutriSim [®]
3- LPS	Rats* were treated with a LD ₁₀₀ single dose of LPS from <i>E. coli</i> serotype 0111:B4
4- LPS plus NutriSim [®]	Rats* were treated with a LD ₁₀₀ single dose of LPS from <i>E. coli</i> serotype 0111:B4, 15 min before NutriSim [®]
5- NutriSim [®] plus LPS	Rats treated with a single dose of NutriSim [®] 15 min before LPS (<i>E. coli</i> serotype 0111: B4)

All rats received a standard diet

Biochemical assays: After 1 h of treatment, the rats were sacrificed by cervical dislocation; their livers were immediately excised and processed to obtain a mitochondrial fraction. Then, the liver was washed in saline solution (0.9% of NaCl) to remove excess of blood and mitochondria were isolated by differential gradient centrifugation. Briefly, the tissue was homogenized with a Teflon[®] pestle PYREX[®] Potter-Elvehjem tissue grinder in 35 mL of cold SHE buffer (250 mM sucrose, 10 mM Tris (pH 7.4), 1 mM EGTA) and maintained at 12°C; once obtained the homogenate, it was centrifuged (600 xg for 5 min at 4°C). The pellet was discarded and the supernatant was centrifuged again (8,000 xg for 10 min at 4°C). The foamy layer at the top of the supernatant was removed and the mitochondrial pellet was washed with SHE buffer (containing 0.1% fatty acid-free serum albumin). Then, the pellet was re-suspended in 0.5 mL of SHE buffer (Moreno-Sanchez and Torres-Marquez, 1991). Protein quantification of mitochondrial membrane was determined by a quantitative colorimetric process for proteins by Folin Reaction based on the Lowry method, in the presence of 0.066% sodium deoxycholate using bovine serum albumin as standard, first the proteins are pre-treated with copper ion in alkali solution, then in the presence of the Folin Reagent a

reduction reaction occurs, turning the end product of this reaction into a blue color. The amount of proteins in the sample can be read at an absorbance of 750 nm (Lowry *et al.*, 1951).

The level of end-products of lipid peroxidation was measured as the result of malondialdehyde (MDA) plus 4-hydroxyalkenals (4-OHA) by a colorimetric method using a LPO Cuvette Based Assay Kit (product No FR 12) obtained from Oxford Biomedical Research, Inc. (Oxford MI, USA). Each measurement was repeated four times. The kit contains a chromogenic reagent (N-methyl-2-phenylindole) which reacts with the lipid peroxidation products MDA and 4-OHA at 45°C yielding a stable chromophore with maximal absorbance at a wavelength of 586 nm, LPO was measured using a Benchmark Plus Microplate Spectrophotometer System, 110/230 v by Bio-Rad and sample analysis was made using the MPM 5.1 PC Software.

Total antioxidant capacity was measured using the Total antioxidant power assay (TA01) reagent kit (obtained from Oxford Biomedical Research, Oxford MI, USA). The combined capacity of serum antioxidants reduces Cu^{+2} to Cu^{+1} which then reacts with the chromogen batocuproin to produce a stable compound with a maximum absorbance at 490 nm. The results are expressed as nM uric acid equivalents.

Membrane fluidity was estimated from the excimer to monomer fluorescence intensity ratio (I_e/I_m) of the fluorescent probe 1,3 dipyrenylpropane (DPyP) incorporated in submitochondrial particles. Briefly, 0.25 mg of mitochondrial protein and 0.1 nmol DPiP were mixed with 10 mM Tris-HCl buffer (pH 7.8). The mixtures were incubated in darkness at 4°C for 5 h, in order to achieve maximal incorporation of the Dpyp to the membranes. The fluorophore was excited at 329 nm and the monomer and excimer fluorescence intensities were read at 379 and 480 nm, respectively; from these readings, the excimer to monomer fluorescence intensity ratio (I_e/I_m) was calculated. Membrane fluidity was expressed as fluorescence intensity ratio of excimer and monomer DPyP (I_e/I_m ratio) and high I_e/I_m ratio indicates a high membrane fluidity. Fluorescence was measured at 24°C on a Perkin Elmer fluorescence spectrometer, LS50B and for sample analysis the FL WinLab 3.0 Software was used, recommended by the manufacturer. Fluorescence corrections obtained from readings of membranes without DPyP were applied to all fluorescence values (Ortiz *et al.*, 2008).

Mitochondrial ATPase activity was determined by quantification of inorganic phosphate (PO_4) released, using colorimetric method described by Sumner (1994). The standard reaction medium (1 mL) contained 125 mM KCl, 40 mM Hepes/KOH (pH 8.0), 0.1 mM EGTA, 3 mM ATP, 5 mM MgCl_2 . The reaction was initiated by the addition of a mitochondrial fraction (1 mg of protein) and quenched by the addition of 100 μL of cold trichloroacetic acid 30% (w/v). Afterwards, the sample was centrifuged for 10 min at 3,500 rpm; and 800 μL of the supernatant was separated and 1 mL of 3.3% ammonium molybdate was added, followed by 100 μL of 10% ferrous sulphate. The reading of the absorbance of the samples was recorded at 660 nm (Sumner, 1944), using the Benchmark Plus Microplate Spectrophotometer System, 110/230 V by Bio-Rad. These analyses were referred to phosphate curve for estimating the enzyme activity in mitochondria.

The transmembrane pH gradient driven by ATP hydrolysis was performed by the measurement of the quenching of 9-amine-6-chloro-2-methoxyacridine (ACMA) fluorescence. Briefly, mitochondrial fractions (2 mg of protein) were incubated at 30°C in a medium (2 mL) containing 125 mM KCl, 20 mM MOPS (pH 7.5), 5 mM MgCl_2 , 0.1 mM EGTA, 1 M ACMA, 3 mM ADP and 5 mM inorganic phosphate. After stabilization of the signal, membrane was energized with 3 mM ATP. ACMA fluorescent quenching is directly related to pH gradient, i.e., the higher values

of fluorescent quenching, the higher values of pH gradient. Fluorescence was measured on a Perkin Elmer fluorescence spectrometer, LS50B, (Rottenberg and Moreno-Sanchez, 1993).

Statistical analysis: All values reported are expressed as Mean±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. The values at $p \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

As shown in Fig. 1a end-products of lipid oxidation (MDA plus 4-OH alkenals) in rat liver mitochondria showed a pronounced increase in response to endotoxin administration ($p < 0.024$) than control group. In contrast, liver mitochondria from the endotoxic group showed a significant reduction of total antioxidant capacity compared with the control group ($p < 0.021$). This data agree with those reported by (Ben-Shaul *et al.*, 1999; Novelli, 1997). The increase of oxidative stress occurred paralleled with the decrease of membrane fluidity in the LPS-treated group ($p < 0.035$) compared with control group (Fig. 1c). These results are consistent with those reported previously by Jackson *et al.* (1989). It is well known that the prime targets of free radical reactions are unsaturated bonds of membrane phospholipids the consequence of these reactions, termed as lipid peroxidation, leads to a loss of membrane fluidity (Chen and Yu, 1994).

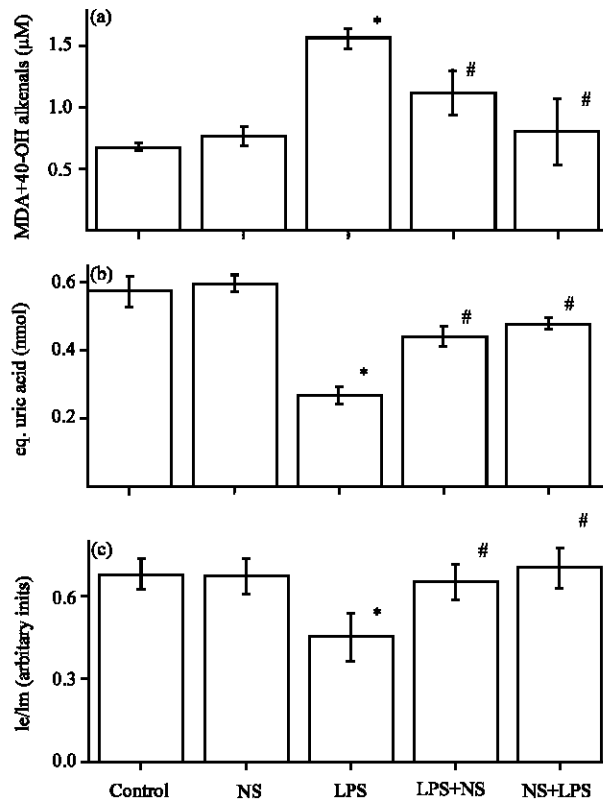


Fig. 1(a-c): (a) End products of lipid peroxidation, (b) Total antioxidant capacity and (c) membrane fluidity in mitochondria from rat liver subjected to the indicated treatments. The bar indicates the Mean±S.E. *indicate a significant difference from the control group ($p < 0.05$). # indicates a significant difference from the LPS group

It has been suggested that liver produces large amounts of ROS species with the purpose of detoxifying against xenobiotic and toxic substances, thus oxidative stress caused by ROS has been shown to be linked to liver diseases, such as hepatotoxicity and other liver pathological conditions (Mehendale *et al.*, 1994; Stohs, 1995). In addition, ROS are known to impair the activity and components of the mitochondrial electron transport chain components and activity (Zhang *et al.*, 1990; Benzi and Moretti, 1995) and damage mitochondrial membranes (Sohal and Dubey, 1994).

It has been demonstrated *in vitro* that endotoxin-induce inhibition of mitochondrial activity, as a secondary response to the infection, coupled with this changes in the enzymatic process of subcellular organelle subsequent to LPS (Schuller *et al.*, 1986; Schumer *et al.*, 1970). Therefore, to determine whether an alteration in enzymatic activity was related to changes in membrane fluidity and oxidative stress, in this work the activity of ATPase both as hydrolytic and as proton-pumping activity in mitochondria was determined. LPS treatment induces a significant decrease of mitochondrial ATPase activity ($p < 0.021$) (Fig. 2a) and transmembrane pH gradient driven by ATP hydrolysis (Fig. 2b) ($p < 0.015$). pH gradient driven by ATP hydrolysis has been extensively used as indication marker of the correct function of the enzyme proton channel and coupling between transport and catalysis (Baracca *et al.*, 2000; Martinez-Cano *et al.*, 2005). Our results suggest that oxidative stress induced by LPS impair ATPase function. In consonance with several reports, suggesting that free radicals cause damage in proteins leading to a loss of enzyme activity (Zhang *et al.*, 1990).

Interestingly, treatment with Nutrisim[®] prior to LPS administration ameliorates significantly the alterations in end-products of lipid oxidation ($p < 0.021$, Fig. 1a), total antioxidant capacity ($p < 0.032$, Fig. 1b), membrane fluidity ($p < 0.011$, Fig. 1c) and ATPase activity ($p < 0.027$, Fig. 2a) and

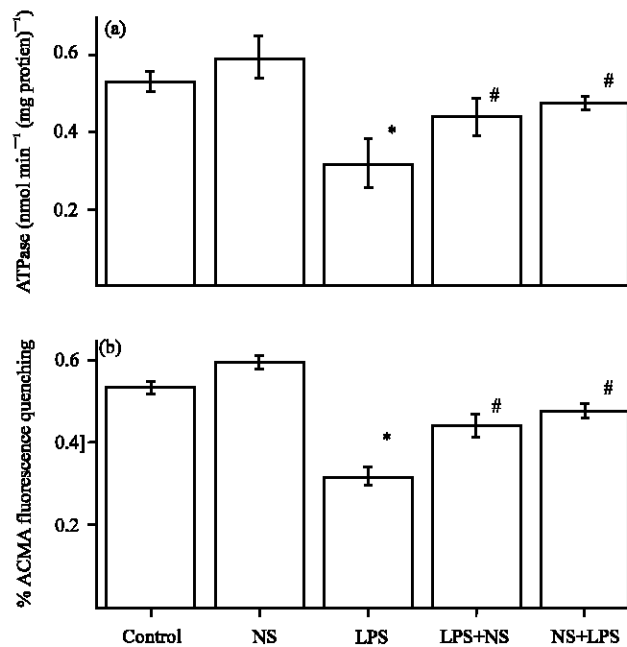


Fig. 2(a-b): (a) ATPase activity and (b) Transmembrane pH gradient driven by ATP hydrolysis in mitochondria from rat liver subjected to the indicated treatments. The bar indicates the Mean \pm S.E. *indicate a significant difference from the control group ($p < 0.05$). # indicates a significant difference from the LPS group

transmembrane pH gradient driven by ATP hydrolysis ($p < 0.015$, Fig. 2b) observed in this septic condition. Treatment with Nutrisim[®] after LPS treatment also attenuates the changes in end-products of lipid oxidation ($p < 0.029$, Fig. 1a), total antioxidant capacity ($p < 0.038$, Fig. 1b), membrane fluidity ($p < 0.010$, Fig. 1c) and ATPase activity ($p < 0.025$, Fig. 2a) and transmembrane pH gradient driven by ATP hydrolysis ($p < 0.023$, Fig. 2b). These data suggest a protective effect of Nutrisim[®] on lipopolysaccharide-induced acute liver injury in rat.

CONCLUSION

In experimental endotoxemia, impaired mitochondrial function and uncoupling of oxidative phosphorylation have been demonstrated. In a previous work we demonstrated that Nutrisim[®] administration attenuates the increase of endotoxin-induced inflammatory mediators, such as TNF- α , IL1 and IL6 (Renovato *et al.*, 2012). The present study shows that Nutrisim[®] preserves ATPase function by attenuating oxidative stress and changes in membrane fluidity. In summary, Nutrisim[®] (a) attenuates endotoxin-induced oxidative stress in liver mitochondria, (b) Preserves mitochondrial membrane fluidity and ATPase activity. The beneficial effects of Nutrisim[®] against oxidative stress and ATPase activity in rat liver mitochondria presented in this study may suggest a potential chemopreventive effect of this compound in sepsis prevention, however further studies are needed to elucidate the mechanisms of these effects.

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