

NutriSim[©] Diminishes an Endotoxin-stimulated Proinflammatory Cytokine Production

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Abstract Lipopolysaccharide, a main component of Gram-negative bacterial endotoxin is the leading cause of sepsis or endotoxic shock. This condition generates tissue hypoperfusion and leads to a state of acute metabolic-circulatory dysfunction and multiorgan systemic failure which contributes to amplify the inflammatory response and resulting in the release of pro-inflammatory cytokines (TNF- α , IL-1 and IL-6). The effect of NutriSim[©] on serum pro-inflammatory cytokine concentrations were investigated in lipopolysaccharide-treated male Wistar rats. Animals were randomized into five groups: control groups receiving a single intraperitoneal injection of physiologic saline solution or NutriSim[©] (10 μ l), respectively; LPS (20 mg/Kg, i.p.); NutriSim[©] treatment 15 min before or after LPS in the same aforementioned dose. The results showed that a single dose of NutriSim[©] prior or before the endotoxic insult diminishes significantly the production of serum TNF- α , IL-1 and IL-6. Therefore NutriSim[©] can modulate an inflammatory response in experimental model of endotoxic shock.

Keywords Nutrisim[©], TNF- α , IL-1, IL-6, Endotoxic Shock, Lipopolysaccharide

1. Introduction

Endotoxic shock is a systemic response to a bacterial infection caused by endotoxins or lipopolysaccharide (LPS)[1]. LPS is a complex glycolipid, composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A, this is the toxic region of the molecule, and responsible for induction of immunostimulatory properties of LPS[2]. LPS is a major component of the outer membrane of Gram-negative (GN) bacteria such as *Escherichia coli*, and one of the most potent microbial initiators of inflammation[3–5].

The inflammatory response to LPS is crucial for protecting the host against pathogenic bacteria, nevertheless uncontrolled and excessive production of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), and interleukin (IL)-1, IL-6, IL-8, and IL-12[6] can be harmful. Cytokines and other mediators may lead to serious systemic complications including microcirculatory dysfunction, liver and kidney damage[7,8], and septic shock with a high

mortality[9].

NutriSim[©], a nutritive supplement that contains arginine, lysine, choline, ammonium chloride, calcium and magnesium is used empirically in the treatment of several degenerative disorders[10]. Previously, we have shown that NutriSim[©] protects against brain damage induced by ischemia-reperfusion in Mongolian gerbils. These effects are partly attributed to its antioxidant action[11]. Free radicals are thought to be involved in the inflammatory process[12], therefore in order to keep evaluating the potential of NutriSim[©], the current study screened anti-inflammatory effect of that nutritive supplement on lipopolysaccharide-induced cytokines production.

2. Materials and Methods

2.1. Materials (Reagents)

The lipopolysaccharide (LPS) of *Escherichia coli*, serotype 0111:B4 was purchased from Sigma Chemical Co. (St. Louis, MO, USA), enzyme-linked immunosorbent assays (ELISA) Kits for cytokine determination specific for rat, was obtained from R&D Systems (Minneapolis, MN, USA) and NutriSim[©] was acquired from Laboratorios BioSim[©] (D.F, México).

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2.2. Animals

Sixty adult male Wistar rats used for this research work were obtained from CIBO-IMSS (Guadalajara, Jalisco, Mexico). The animals weighed 200 - 250 g, maintained on a 12h/12h light-dark cycle at ($22 \pm 2^\circ\text{C}$), fed with standard diet (Chow Purina) and water *ad libitum*.

2.3. Experimental Design

Animals were randomly assigned to five experimental groups as shown below.

Group Control; treated with physiologic saline solution administered by intraperitoneal (i.p.) injection

Group NutriSim[®]; received a single dose (10 μl /100g of body weight, i.p.) of NutriSim[®].

Group LPS; received a LD100 dose of LPS (20mg/Kg of body weight, i.p.).

Group LPS plus NutriSim[®]; received a LD100 dose of LPS (20mg/Kg of body weight, i.p.), 15 min before NutriSim[®].

Group NutriSim[®] plus LPS; NutriSim[®] was administered with a dose of NutriSim[®] (10 μl /100g of body weight, i.p.) 15 min before LPS.

Animals were sacrificed at 15, 30 and 60 min after the treatment; blood was collected immediately and centrifuged for 10 minutes, at 3000 rpm for collection of serum. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard [13].

2.4. Cytokines Quantifications (ELISA)

TNF- α , IL-1 β and IL-6 levels in the serum were quantified using commercially available ELISA kits specific for rat TNF- α (Genzyme, Cambridge, MA, USA), IL-1 β and IL-6 (both from Endogen, Woburn, MA, USA). The sensitivity of the assays for TNF- α , IL-1 β and IL-6 was 5 pg/ml, 12 pg/ml and 15 pg/ml, respectively. Absorbance at 405-414 nm was measured at a maximum of 15 min after the addition of substrate, using a UV-Vis spectrophotometer (BIO-RAD 550).

2.5. Statistical Analysis

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 Newman-Keuls t test; $p \leq 0.05$ was considered significant.

3. Results

3.1. Serum Cytokines Levels

Basal levels of serum TNF- α , IL-1 β and IL-6 were detected in saline-treated rats, respectively. Pre-treatment of rats with NutriSim[®] alone did not affect the serum level of these cytokines (Figures 1-3). The concentration of TNF- α in serum rapidly increased, reaching a peak at 30 min after injection of LPS, then diminishes. This increase was found to

be statistically significant in comparison with the control group ($p < 0.01$). NutriSim[®] prevents excessive expression of TNF- α in response to LPS ($p < 0.01$), however the effect was higher when injected prior LPS administration (Figure 1).

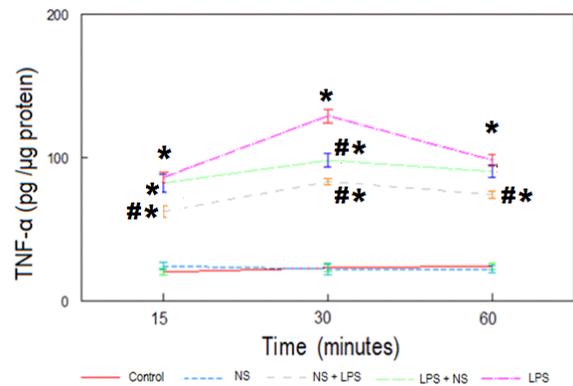


Figure 1. Time course of serum TNF- α levels from the indicated rat groups. Each point represents the mean \pm SEM. * $P < 0.01$ vs. control groups. # $P < 0.01$ vs. LPS group

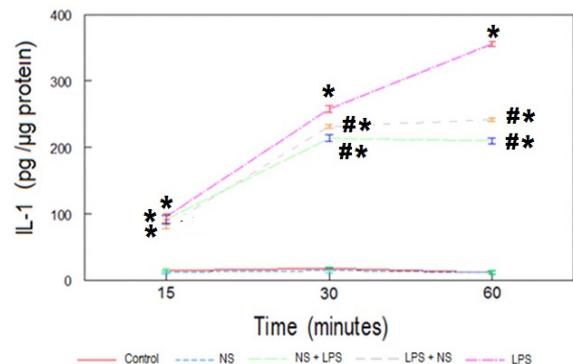


Figure 2. Time course of serum IL-1 β levels from the indicated rat groups. Each point represents the mean \pm SEM. * $P < 0.01$ vs. control groups. # $P < 0.01$ vs. LPS group

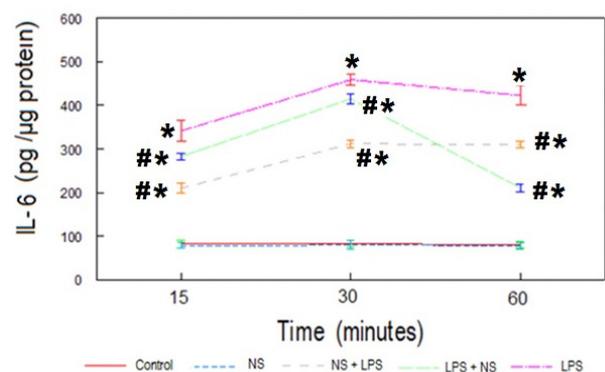


Figure 3. Time course of serum IL-6 levels from the indicated rat groups. Each point represents the mean \pm SEM. * $P < 0.01$ vs. control groups. # $P < 0.01$ vs. LPS group

LPS challenge caused a marked rise in the level of IL-1 β compared to control group; treatment with NutriSim[®] after and before the injection of a LD100 of LPS diminishes significantly the serum IL-1 β values during the course of the experiment ($p < 0.05$). This was more evident at 60 minutes after LPS treatment. (Figure 2)

IL-6 release also occurred very rapidly and was

significantly increased at 15 min, peaking at 30 min post-injection of LPS ($P < 0.01$ vs. control). Experimental groups LPS plus NutriSim© and NutriSim© plus LPS showed a significant decrease in the serum IL-6 concentration ($p < 0.01$, Figure 3).

4. Discussion

We found that intraperitoneal administration of lipopolysaccharide evoked a robust systemic response that induced a stereotypical cytokine release. Macrophages are target cells of LPS [14]. After interaction with an endotoxic bacterium, the innate immunity is rapidly triggered, primarily via activation of toll-like receptor 4 (TLR-4) [15]. Activation of TLR-4 induces a multitude of pro-inflammatory cytokines via activation of transcription factors, nuclear factor κ B (NF- κ B) [16]. This prompt response provides a favorable environment for the synthesis and upregulation of both IL-1 β and IL-6, which together contribute to the perpetuation of the inflammatory challenge.

The pro-inflammatory response observed in septic shock is mediated by counter-regulatory molecules which attempt to restore the immunological equilibrium, amongst counter pro-inflammatory molecules include TNF, IL-1; alternatively, IL-10 an anti-inflammatory cytokine modulates the cascade and synthesis of inflammatory factors by lymphocytes T and macrophages [17].

Also the rapid increase in TNF- α following LPS, which is already present after 15 minutes, promotes synthesis of other cytokines and the initiation of the acute-phase response, chemokine release and oxidative stress [18]. Interestingly, the production of the reactive oxygen and nitrogen species is an inherent property of activated immune cells. Inflammation can lead to oxidative stress and conversely. Thus, oxidative stress and inflammation are involved in a self-perpetuating cycle.

It is known that a deficiency of dietary amino acids impair immune functions, increasing the susceptibility to an infectious disease. The role of amino acids such as arginine and lysine is very important in the immune response; they regulate the activation of T and B lymphocytes, cellular redox status and production of cytokines [19] which can be cytotoxic substances in pro-inflammatory process.

In this work we found that administration of NutriSim© 15 minutes prior or after LPS challenge diminishes significantly the level of serum cytokines. Thus supplementation with a nutritional substance containing specific amino acids possibly enhances immune system response. On the other hand, anti-inflammatory effect could be due to L-arginine, a semi-essential amino acid, which has been describe to play an important role in stress situations like sepsis [20]. Additionally, it has been demonstrate that arginine diminishes lipid peroxidation in patients with diabetes mellitus [21]. It has been demonstrated that patients with severe sepsis shown low levels of arginine amongst others like citrulline and ornithine [22]. This could suggest

that availability of arginine supplementation could be useful as a probable potential therapeutic in cases on septic shock, though it hasn't been demonstrated the relation between low levels of arginine and severity of sepsis.

At this regard, it has been reported that natural antioxidants such as melatonin [23], quercetin [24], luteolin [25] and other antioxidants (butylated hydroxyanisole, tetrahydropapaveroline, nordihydroguiauretic acid, 10, 11-dihydroxyaporphine) [25] are potent inhibitors of the LPS-induced production of TNF- α production *in vivo*.

Choline is a precursor of neurotransmitter acetylcholine and an essential nutrient; it has also been demonstrated *in vitro* that choline inhibit TNF release in macrophages, and this might suggest that choline may be have anti-inflammatory activity [26], although the efficiency of choline should be further investigated.

NutriSim© administration in the absence of LPS does not altered the serum cytokines levels, which might suggest that this supplement does not exert a negative effect on the immune system.

5. Conclusions

It has been already demonstrated that LPS increase the cytokines production in immune cells and it can modulate both pro- and anti-inflammatory response; in this work we demonstrate that the use of a nutritional supplement such as NutriSim©, can modulate an inflammatory response in experimental model of endotoxic shock. However, the mechanism of action by which this supplement exerts its beneficial effect is still unclear. Whether our findings may be of therapeutic value needs to be further investigated.

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REFERENCES

- [1] Xu H, Gonzalo JA, Pierre Y St, Williams IR, Kupper TS, Cotran RS, Springer TA, Gutierrez-Ramos JC. 1994. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *J. Exp. Med.*, 180: 95-109
- [2] Rietschel ET, Brade L, Brandenburg K, Flad HD, Jong-Leuveninck J, Kawahara K, Lindner B, Loppnow H, Lüderitz T, Schade U. 1987. Chemical structure and biological activity of bacterial and synthetic lipid A. *Rev. Infect. Dis.*, 9(Suppl. 5): 527-536.
- [3] Christian R. and Raetz H. 1990. Biochemistry of endotoxins. *Ann Rev. Biochem.*, 59: 129-170
- [4] Jonathan C. 2002. The immunopathogenesis of sepsis. *Nature*

- 420: 885–891
- [5] Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F. 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB J.* 8: 217–225
- [6] Cavaillon JM, Conquy MA. 1998. Sepsis, Pathophysiological Role of Pro- and Anti- Inflammatory Cytokines in Sepsis.2, Kluwer Academic Publisher, Boston
- [7] Hewett JA, Roth RA. 1993. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol. Rev.* 45: 381–404
- [8] Hirata K, Kaneko A, Ogawa K, Hayasaka H, Onoe T. 1980. Effect of endotoxin on rat liver. Analysis of acid phosphatase isozymes in the liver of normal and endotoxin-treated rats. *J. Tech Meth. Path.* 43: 165–171
- [9] Ulevitch RJ, Tobias PS. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* 13: 437–457
- [10] Miranda Riestra A. 2004. Estudio bioquímico del efecto protector del NutriSim© en cerebros de mongolian gerbils (*Meriones unguiculatus*) sometidos a isquemia-reperfusion Thesis. Universidad de Guadalajara. México.
- [11] Rolando R. Dávalos, Luis A. R. Tirado, José J. H. Andalón, Erika D. G. Renovato, Irma E. V. Brizuela, Cruz-Ramos J. A., Genaro G. Ortiz, Fermin P. P. Moisés. 2011. NutriSim© Protects-Against Apoptosis in Hippocampus Induced by Transient Ischemia in Mongolian Gerbils (*Meriones Unguiculatus*). *J. Med. Sci.* 4: 40-46
- [12] Online Available: <http://www.biostrategies.com/kpmsgendo.pdf>
- [13] Chatterjee S, Premachandran S, Raghavendra S, Bagewadikar, Bhattacharya S, Chattopadhyay S, Poduval T.B. 2006. Arginine metabolic pathways determine its therapeutic benefit in experimental heatstroke: Role of Th₁/Th₂ cytokine balance. *Nitric Oxide.* 15:408-416
- [14] Bradford M. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72: 248–256
- [15] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162: 3749-3752
- [16] Andreakos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM. 2004. Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood.* 103: 2229-2237
- [17] Cohen J. 2002. The immunopathogenesis of sepsis. *Nature.* Vol 420, p 885-891
- [18] Remick DG, Strieter RM, Eskandari MK. 1990). Role of tumor necrosis-alpha in lipopolysaccharide-induced pathologic alterations. *Am. J. Pathol.* 136: 49 – 60
- [19] Peng Li, Yu-Long Yin, Defa Li, Sung Woo Kim and Guoyao Wu. 2007. Amino acids and immune function. *British Journal of Nutrition*, 98 , pp 237-252.
- [20] Sewerinek E, Abe M, Chen L, Ortiz GG, Reiter J. 1995. Oxidative changes in the liver, brain and lens of lipopolysaccharide-treated rats. *Arch Med Res* 26: 5109 –5115.
- [21] Lubec B, Hayn M, Kitzmuller E, Vierhapper H, Lubec G. 1997. L-arginine reduces lipid peroxidation in patients with diabetes mellitus. *Free Rad. Biol. Med.* 22: 355–357
- [22] Eugui EM, DeLustro B, Rouhafza S, Ilnicka M, Lee SW, Wilhelm R, Allison AC. 1994. Some antioxidants inhibit, in a co-ordinate fashion, the production of tumor necrosis factor-alpha, IL-beta, and IL-6 by human peripheral blood mononuclear cells. *Int Immunol.* 6: 409-22
- [23] Bitzer-Quintero OK, Ortiz GG, Ruiz-Rizo L, Torres-Mendoza BM, Vazquez-Valls E, Rodriguez-Perez M. 2005. Effects of melatonin on plasma levels of TNF- α , IL-1 and IL-6 in mice after lipopolysaccharide administration *Biol. Rhythm Res.* 36(1/2): 159– 68
- [24] Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C. 2006. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. *Clin. Vacc. Immunol.* 13: 319-28
- [25] Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, Roussos C. 2001. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. *J. Pharmacol. Exp. Ther.* 296: 181-187
- [26] Rowley TJ., McKinstry A., Greenidge E., Smith W., Flood P. 2010. Antinociceptive and anti-inflammatory effects of choline in a mouse model of postoperative pain. *British Journal of Anaesthesia* 105 (2): 201–7