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Effect of NutriSim© and the Interactive Response of Pro-inflammatory and Anti-inflammatory Cytokines in a Model of Septic Shock Induced by *E coli* Serotype 0111:B4

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Abstract The biological effects and possible mode of actions of anti and pro-inflammatory cytokines on the evolution of sepsis and of its most severe complication, the septic shock, were exposed. These data were analyzed in order to clarify the complex pathogenic mechanisms taking place in animal models of sepsis and human sepsis. This study makes possible the development of new drugs and other effective and safe therapeutically procedures for the treatment of a severe disease causing a high morbidity and mortality worldwide. The complex interactions occurring among the diverse pro-inflammatory cytokines were stressed, as well as the interactions with other mediators of the so-called systemic inflammatory response. The dual behavior of the actions of some cytokines, occasionally carriers of opposed effects, as well as their actions depending on the period of time in which they were administered, were emphasized. Another significant characteristic was that they frequently varied, and that there was no correspondence between the results of the effects of the cytokines in humans and those obtained in experimental animal models of sepsis or endotoxic shock. The effect of NutriSim©, a nutritional supplement, on serum anti and pro-inflammatory cytokines levels using an experimental model of endotoxic shock were studied. The results exhibited that a single dose of NutriSim© prior the endotoxic insult diminishes significantly the production of serum TNF-α, IL-1, IL-6, IL-4 and IL-10 cytokines.

Keywords NutriSim©, TNF-α, IL-1, IL-6, IL-10, IL-4, Septic Shock, Lipopolysaccharide

1. Introduction

Septic Shock

Sepsis and septic shock are responsible for a high morbidity, are also the most common cause of death in intensive care units. Historically, the mortality associated with sepsis was maintained between approximately 50 and 75% in the patients, but with the advent of antibiotic therapy was reduced to a range of 30 to 50%. Despite this progress, recent estimates indicate the existence of 750 000 cases of severe sepsis per year in the U.S. [1]. But the number is gradually increasing in 9.5% of the reported cases and the mortality rate remains high. Given its importance, the role of pro-inflammatory cytokines in the pathogenesis of septic shock will be addressed, because the modulation of these mediators is the most important and promising strategy being investigated as a therapeutic option to reduce morbidity and characterizing high mortality in septic shock [2].

It is known that sepsis syndrome occurs due to bacteria and other microorganisms that cause infectious focus (pneumonia, abscesses, etc.) Or bacterial exotoxins constituents released in the local or systemic host environment. These constituents include bacterial cell wall components such as endotoxins in Gram-negative bacteria, teichoic acid and other antigens in Gram-positive bacteria and bacterial DNA [3]. These products stimulate the generation of pro-inflammatory cytokines both locally and systemically, which exert multiple effects, such as stimulating the production and release of other

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pro-inflammatory mediators. Among them, the most important pro-inflammatory cytokines known that exert with both synergistic effects by stimulating the cascade of inflammatory mediators are: tumor necrosis factor alpha (TNF-alpha) and interleukin 1beta (IL- 1beta), triggering the so-called systemic inflammatory response syndrome (SIRS) due to an infection [4]. The next phase in the cytokine response to infection is an opposite response to inflammatory activity, which is exerted by inhibitors such as receptor antagonist of (IL-1) and TNF receptor inhibitor and some anti-inflammatory cytokines such as IL-4, IL-6, IL-8, IL-9, IL-10, IL-11 and IL-13, which produces the compensatory anti-inflammatory response syndrome (CARS). It is precisely the balance between these cytokines in different periods of time, which determines the clinical manifestations (figure 1) and the successful or fatal outcome in sepsis and septic shock [5].

Gram negative bacteria release LPS to the bloodstream, inducing a local and systemic effect. LPS is known to stimulate immune response thus the release of pro-inflammatory mediators such as tumor necrosis factor Alfa (TNF-α) and interleukin 1-beta (IL-1β), leading to a systemic inflammatory response syndrome due to infection. The next phase produces the compensatory syndrome, where the balance between anti and pro inflammatory mediators will determine the clinical manifestations of sepsis.

2. Pro-inflammatory Cytokines

TNF-α

This molecule was first identified in the pathogenesis of septic shock; from a structural point of view is a 17kDa protein produced predominantly by macrophages and has the ability to activate monocytes, macrophages, neutrophils and induce the production of acute phase proteins through the IL-6 pathway [6].

Two different cell surface receptors have been described for the TNF-α; the type I having a molecular weight of 55 kDa and type II with a molecular weight of 75 kDa. The stimulation of these receptors leads to cytotoxicity, activation of factor nuclear kappa B (NF-kB) as well as the expression of adhesion molecules on endothelial cells [7]. Both receptors are also present in soluble form and retain their affinity for TNF-α. Soluble receptors competing with cell surface receptors for binding to free TNF-α, because it is inactivated when bound or soluble binds receptor, the generation of these receptors actually represents an anti-inflammatory response mechanism [8]. Numerous studies have demonstrated the presence of high levels of TNF-α in clinical sepsis and septic shock [9]. (Table 1)

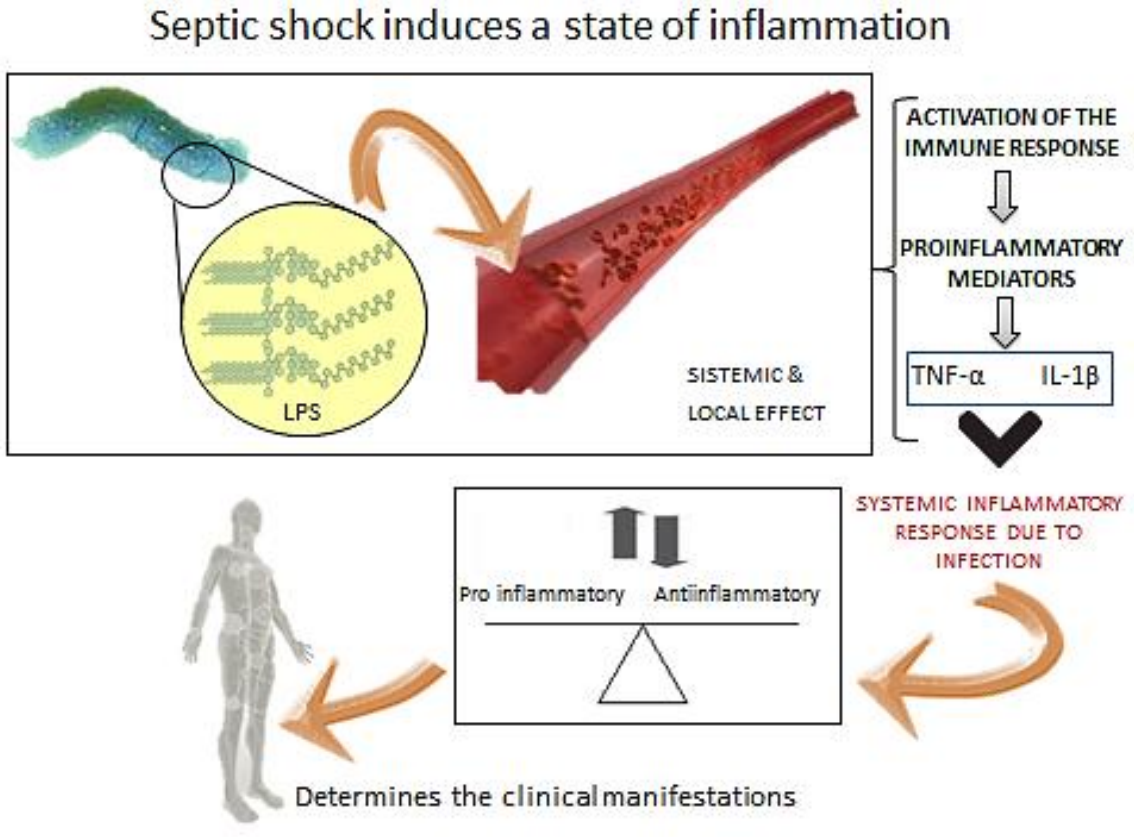

Figure 1. Septic shock induces a state of inflammation

Table 1. Pro-Inflammatory Cytokines

Type	Produced by	kDa Weight	Effect
Tumor Necrosis Factor-alpha	Macrophages	17 kDa	Activates Monocytes, macrophages, neutrophils and the production of acute phase proteins.
Interleukin-1beta	Mononuclear phagocytes and neutrophils	17 kDa	Main initiator of the cascade of cytokines
Interleukin-6	Monocytes, macrophages, endothelial cells, fibroblasts, lymphocytes B and T.	21 kDa	Control of inflammation by inducing production of acute phase proteins.

Table 2. Administration of Recombinant TNF-Alpha in Animal Models

Symptoms	
	Fever
	Disseminated Intravascular Coagulation
	Pulmonary Edema
Others	
	Lactic Acidosis
Administration of recombinant TNF-alpha in humans	
Symptoms	
	Hypotension
	Fever
	Chills
	Tachycardia
Others	
	Metabolic Acidosis
	Leukopenia
	Thrombocytopenia
	Decreased systemic vascular resistance

Endotoxin (*E. coli*-LPS) administration to animal models manifested an increased in serum TNF-α, which is associated with cardiovascular and metabolic disorders, similar to those that occur in sepsis and septic shock in human [10]. Animal models, administration of recombinant TNF-α causes fever, lactic acidosis, disseminated intravascular coagulation, pulmonary edema and death [11]. The behavior of the cardiovascular system in these animals is similar to that of septic shock in humans and is characterized by hypotension and decreased of systemic vascular resistance. Administration of TNF-α to human induces fever, chills, metabolic acidosis, leukopenia, thrombocytopenia, tachycardia and hypotension with decreased peripheral vascular resistance [12]. (Table 2)

Interleukin 1-β

Is a polypeptide of 17kDa produced by mononuclear phagocytes and neutrophils. Currently they are recognized to be the main mediators present, in addition with the TNF-alpha, in the cascade of cytokines released in sepsis[13]. Most of the biological effects of TNF-alpha induces IL-1beta, which, when injected into animals and humans produces fever chills, heart and vascular disorders with leukopenia, thrombocytopenia, bleeding, and pulmonary edema; the release of IL-1beta is increased in experimental models of sepsis and septic shock in animals (LPS) and humans, and these high levels of both cytokines was also correlated with a high mortality. As the case with endotoxin, administration of TNF-alpha to humans and animals is associated with increased circulating levels of IL-1beta. Detected higher levels of IL-1 beta have been observed in patients with meningococcal sepsis and this has been correlated with the severity of meningococcemia, shock and dead [14]. (table 3)

Table 3. Administration of IL-1 Beta in Humans and Animals

Symptoms	
	Fever
	Chills
	Heart and vascular disorders
	Bleeding
	Pulmonary Edema
Others	
	Leukopenia
	Thrombocytopenia

Interleukin-6 (IL-6)

IL-6 is a 21 kDa protein produced by activated monocytes, macrophages, endothelial cells, fibroblasts and B and T lymphocytes. When this cytokine is administered to animals produces mild clinical symptoms (fever and chills), but these are not accompanied by hemodynamic changes or toxicity observed with TNF-α and IL-1β [15]. Most of the activities of IL-6 are associated with the control of inflammation as a result of its potent ability to induce production of acute phase proteins in the liver, which are essentially protecting and limiting the inflammatory process through its anti-protease activity and sequestering capacity [16]. This is confirmed by the fact that injection of IL-6 by LPS protects from death in experimental endotoxic shock [17]. The IL-6 also free receptor antagonist and IL-1β in that context, it is interesting that this has recently been identified as a product of hepatocytes and regulated by pro-inflammatory cytokines, as well as the acute phase proteins (APP) as alfa1antitripsina and alpha1-acid glycoprotein, among others, by which the receptor antagonist of IL-1β in fact today is regarded also as a APP; they protect-against endotoxin and can even reduce the lethality of TNF-α [18]. These results can explain why IL-6 has been shown to have protective activity in infections and septic shock models. However, in contrast to these beneficial effects, IL-6 can cause bone resorption, muscle wasting, anemia and can stimulate the production of neutrophils to platelet activating factor (APP) and the superoxide anion [19-21].

IL-6 does not activate endothelial cells but in the presence of its soluble receptor, which is found in the plasma, induced chemokines like monocyte chemoattractant protein (MCP- 1) and IL-8 stimulate the recruitment of neutrophils. Also deleterious effects of IL-6 have been detected *in vivo* in experimental models of ischemia-reperfusion induced in rat [22]. These adverse effects produced by IL-6 may be the explanation why many researchers have pointed out that elevated circulating levels of this cytokine correlate with the severity of sepsis and prognosis of death in patients, many consider the IL -6 cytokine also as a pro-inflammatory (dualism) [23].

3. Anti-inflammatory Cytokines

Currently considered the most important anti-inflammat ory cytokines IL-10, IL-4, IL-6, IL-13, the granulocyte colony stimulating factor-macrophage (GM-CSF), IFN-alpha and transforming growth factor- beta (TGF-β) for the role they play in the pathogenesis of sepsis. It is noteworthy that several cytokines exert a dual role in septic processes and act as pro-inflammatory or anti-inflammatory in the following influencing factors: a) The amount of cytokines present; b) The nature of the target cells; c) The type of triggering agent that acts on target cells; d) The time period of exposure to target cells; e) The experimental model used[24]. (table 4)

Table 4. Anti-inflammatory Cytokines

Type	Produced by	Action
Interleukin 10 (IL-10)	T helper type 2 cell	Inhibit the production of pro-inflammatory cytokines: - T helper type I -Monocytes -Macrophages - Neutrophils
Interleukin 4 (IL-4)		Inhibit the synthesis of pro-inflammatory cytokines
Lipopolysaccharide (LPS)	Component of the outer membrane of Gram negative bacteria.	1.- Microbial initiator of inflammation 2.- Activates: - Monocytes - Macrophages

Interleukin 10 (IL-10)

This was the first anti-inflammatory cytokine evaluated clinically for sepsis therapeutic application [25]. It was initially identified as a product of T helper type 2 cells in mice, which inhibit the production of pro-inflammatory cytokines by Type 1 T helper cells [26]. Exerts its anti-inflammatory on monocytes/ macrophages actions, Neutrophils and T-cells exhibited initially the mortality inhibition in experimental endotoxic shock [27].

Consistent with this, it has been demonstrated that mice IL-10-deficiency are much more vulnerable to mortality induced by lipopolysaccharide (LPS) [28] while also was demonstrated an increased mortality when endogenous IL-10 was blocked by monoclonal anti-IL-10 [29]. IL-10 administered in an experimental model of sepsis induced by cecal ligation and puncture in mice, did not reduce the mortality or morbidity in experimental sepsis, which appears to be caused by an immunosuppressive effect of IL-10 which explains why infection persists in septic animals, although they administer *a priori* antibiotic therapy. Experience with the administration of human IL-10 has been limited to experimental endotoxic-induced in healthy volunteers, in that pretreatment with IL-10 induces complex responses in body temperature, TNF-α levels, IL-6, IL-8 and the IL-1, when compared with the control group Decreased accumulation was also detected in neutrophils in the lung. However, when treated with IL -10 after administration of endotoxin, the febrile response, the cytokine release, and neutrophil accumulation were not modified [30]. A similar behavior of the IL-10 was observed with relation to hemodynamic parameters (heart rate and pressure) which were not modified by the administration of IL -1 after endotoxin [31].

Interleukin-4 (IL-4)

This has a potent anti-inflammatory activity and is capable of inhibiting the synthesis of pro-inflammatory cytokines and have been demonstrated the ability of reducing mortality in several models of septic or endotoxic shock, Mice pretreated with IL-4 survive intraperitoneal injection (ip) of live *E coli* (10 colony forming units and bacteroides fragilis (10CFU), which killed 90% of the mice pretreated with the cytokine. However, it was also demonstrated that pretreatment with IL-4 prior to the induction of sepsis had protective effect, instead when IL-4 is administered during

the first infection mortality is increased [32] which argues the importance of selecting the time period and the most suitable of administration to produce the desired effect (in this case, reducing mortality) and also illustrates how this important factor influencing the dual behavior (effectiveness or lack of effect) of each of the different cytokines in the time septic processes [33].

Lipopolysaccharide (LPS)

Bacterial endotoxin lipopolysaccharide (LPS), a complex glycolipid, is composed of a hydrophilic polysaccharide and a hydrophobic domain known as lipid A. LPS is a major component of the outer membrane of Gram negative bacteria and one of the most potent microbial initiators of inflammation [34]. It has been shown that LPS activates monocytes and macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and interleukin (IL)-1, IL-6, IL-8, and IL-12 also anti-inflammatory cytokines (IL-10, IL-4 etc. Macrophages also secrete, in response to LPS, a wide variety of other biological response mediators including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide [35].

Production of these inflammatory cytokines and mediators by monocytes / macrophages contributes to the efficient control of growth and dissemination of invading pathogens [36]. However, excessive and uncontrolled production of these inflammatory cytokines and mediators may lead to serious systemic complications including microcirculatory dysfunction, liver and kidney damage [37], and septic shock with a high mortality [38].

4. NutriSim© & Pro-and Anti-inflammatory Cytokines

Previously we have shown that NutriSim©, a nutritive supplement used empirically in the treatment of several degenerative disorders protects against brain damage induced by ischemia-reperfusion in *Mongolian gerbils*. These effects are partly attributed to its antioxidant action [39]. Also we demonstrated that administration of NutriSim© 15 min before LPS decrease the level of TNF-α, IL-1 and IL-6 in serum compared to group LPS exhibited a reduction of the levels of secreted TNF-α and IL-6 in serum 30 min after the treatment with LPS. Intraperitoneal administration of lipopolysaccharide stimulates in macrophages cytokines production which conduces to an inflammatory and non-controlled response [40].

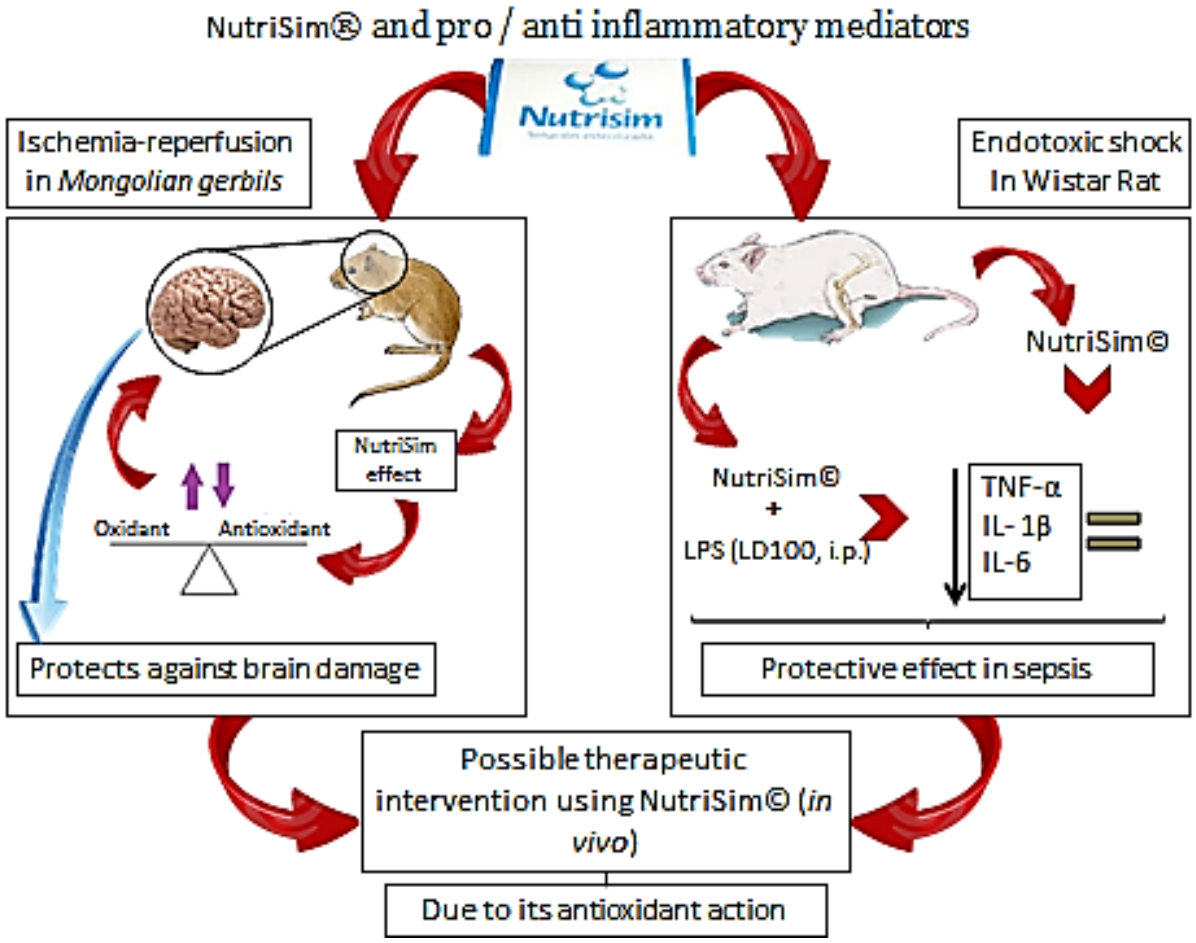

Figure 2. NutriSim and pro / anti- inflammatory mediators

In the first paper [41] we demonstrated that administration of NutriSim© 15 min before LPS decrease the level of TNF-α, IL-1 and IL-6 in serum compared to group LPS. In this paper we showed a reduction of the levels of secreted TNF-alfa and IL-6 in serum 30 min after the treatment with LPS. This data possibly refers to the role of inhibitors, like the antagonist receptor of IL-1 (IL-1), or the effect of some anti-inflammatory cytokines like IL-6 itself, which plays an important role as an anti-inflammatory cytokine in the compensatory anti-inflammatory response syndrome. On the other hand in the second paper exhibited that the basal low levels of serum IL-4 and IL-10 these cytokines were detected in saline-treated rats and pretreatment with NutriSim© alone, it does not altered these values [42]. After injection of LPS, the concentration of IL-4 in serum rapidly increased, reaching a peak at 30 and 60 minutes (Figure 2). This increase was found to be statistically significant in comparison with the control group ($p<0.01$). Treatments with NutriSim© prevents excessive expression of IL-4 in response to LPS ($p<0.01$). LPS challenge caused a marked rise in the level of IL-10 compared to control groups; NutriSim© treatments whether before or after the injection of a LD100 of LPS exhibited significantly lower serum levels of IL-10.

This data possibly refers to the role of inhibitors, like the antagonist receptor of IL-1 (IL-1 ar), or the effect of some anti-inflammatory cytokines like IL-6 itself, which plays an important role as an anti-inflammatory cytokine in the compensatory anti-inflammatory response syndrome.

Acute kinetic plasma levels of pro and anti-inflammatory molecules have been extensively studied [43]. As a first step in predominant pro-inflammatory mediator's effect [44], downstream phenomena predominate anti-inflammatory associated with a decrease in nitric oxide production [42].

The role of TNF-α and IL -1 beta in sepsis appears to be the trigger, but both lack of pathogenic value subsequently, as are well inhibited pathophysiologically through the soluble receptors and of IL-1 ra, respectively. The soluble tumor necrosis factor receptors I and II (sTNFR-I, sTNFR-II) and IL -1 have great prognostic value and their levels are related to the development of multiple organ dysfunction syndrome (MODS) and each organ failure [45]. Elevated levels of IL-10 and TGF- beta are also associated with mortality, although so later. MODS, show high levels of anti-inflammatory cytokines such as those of IL-6, soluble type I and type II receptors of TNF, IL-1 and the receptor for IL-2. For each organ failure, is to highlight the role that TNF-alfa plays in the development of acute renal failure and having the IL-1beta in discriminating patients with circulatory failure. It is also important to note that the appearance of IFN-, IL-10 and TGF-beta after the first days of sepsis pathophysiology has great value, which can be used to better understand the intimate mechanisms of this process [46].

Future therapeutic interventions (including NutriSim©) should note that sepsis is a dynamic process and consider the time of disease progression with respect to immune response. In this case treatment should be individualized and possibly adapted to balance pro-and anti-inflammatory. The characteristics of the immune process depend on the genetic polymorphism of the person who has the disease, the time when we are in the evolution of the same and the particular characteristics of the pathogen responsible for the infection.

NutriSim is a nutritive supplement empirically used in the treatment of several degenerative disorders, in an experimental model in Mongolian gerbils its administration demonstrated a protective effect against bran damage, in other study the use of this supplement in a model of endotoxic shock in Wistar rat using lipopolysaccharide at a lethal dose (LD100) administered intraperitoneally (i.p.) diminished the levels of pro-inflammatory cytokines (TNF-a, IL-1B and IL-6), while administered alone did not change the basal concentrations, showing a protective effect in sepsis. Both models have demonstrated a possible therapeutic intervention due to its antioxidant action.

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 state, as demonstrated before by our investigation group, that the use of a nutritional supplement such as NutriSim©, can modulate both pro- and inflammatory response in experimental model of endotoxic shock. Though we can suspect it acts at a cellular level, inducing beneficial changes in the presence of endotoxins. However, the mechanism of action by which this supplement exerts its beneficial effect is still unclear. Whether our findings may be of therapeutic value, it needs to be further investigated.

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