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Original article

Antioxidant activity of tryptophan in rats under experimental endotoxic shock

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ABSTRACT

Tryptophan (TRP), the precursor of the scavenger or immunomodulator molecules melatonin (MLT) and picolinic acid, can be found in the diet; and could be an alternative nutritional supplement used to regulate the immune response in the generation of free radicals. In an experimental model, the systemic administration of lipopolysaccharide (LPS), to promote the synthesis of pro-inflammatory cytokines, reactive oxygen species, and antioxidant enzymes, was performed on adult female, pregnant and lactating rats fed with a diet of TRP content (0.5 mg/100 g protein). Lung tissue was evaluated for levels of the products of lipoperoxidation (LPO's), malonaldehyde (MDA) and 4-hydroxy alkenals (4-HDA); nitrites (NO2), glutathione peroxidase (Gpx) enzyme activity, and the serum concentration of interferon-gamma (IFN-γ), which were measured in the following groups: control (CTRL), LPS, MLT, TRP, LPS plus MLT (LPS + MLT), and LPS plus TRP (LPS + TRP). Results showed that the lung tissue levels of MDA and 4-HDA in the LPS + TRP group were significantly lower than in the TRP group. Statistically significant differences were not observed in nitric oxide levels among the groups LPS + MLT and LPS + TRP compared to the group under endotoxic shock (LPS). The Gpx enzyme activity was modified in the LPS + MLT vs the LPS group, but the difference was not statistically significant. The LPS + MLT group showed a smaller serum concentration (98%) of IFN-γ than the LPS group. Statistically significant differences were not observed among the animals of the LPS + TRP and the LPS groups.

1. Introduction

Tryptophan (TRP) is an essential amino acid for the biosynthesis and structure of proteins that are implicated in a variety of metabolic processes, and it is also the main precursor of serotonin (5-HT), melatonin (MLT) (secreted by the pineal gland), and niacin [1–4].

Nutritional habits in third world countries affect dietary TRP supplies, since diets are primarily based on cereals, wheat and corn, which are TRP deficient with a high concentration of neutral amino acids that compete with TRP to be transported by albumin into the nervous system [5,6].

A TRP-deficient diet leads to a negative TRP balance or a state of malnutrition in the organism, as well as a reduction in the final degradation of TRP metabolites, like serotonin, picolinic acid, niacin, and MLT [7]. While the degradation products of MLT participate in the activation and suppression of immune responses, picolinic acid acts as a costimulation signal in IFN-γ synthesis during the process of macrophage activation [8]. The 5-HT inhibits the expression of the major complex of histocompatibility class-II (MHC-II) murine molecules that are induced by the same IFN-γ [9–11], which is considered a potent immunomodulator with antiproliferative and antineoplastic effects that is able to induce the expression of the indoleamine-2,3-dioxygenase (IDO) and is a key enzyme in the TRP metabolic process, since TRP breaks it down toward kynurenines (kyn) in the pathway [10,12,13].

The pineal gland produces MLT, a neurohormone with a wide range of functions [14,15]. One of its main abilities is to scavenge

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and reduce free radicals during the endotoxic shock process [16,17].

Recruitment of neutrophils to extravascular sites of bacterial infection is essential to host defenses in severe infection; however, local defenses may be inadequate and both bacteria and their products enter into the bloodstream and produce a generalized inflammatory response. This response results in sepsis or endotoxic shock with the release of proinflammatory molecules [18–20]. It has been shown that high amounts of proinflammatory cytokines in the blood are able to trigger a "state of shock" with consequent damage to organs [21–23].

Nevertheless, the effect of TRP as a free radical scavenger has not been tested. A diet based on high TRP concentrations is economical and innocuous, and could be an important alternative treatment in patients with endotoxic shock. Although the direct relationship between nutritional management and endotoxic shock has not been well established, numerous animal studies suggest that nutraceutics (TRP-based diet) may affect the immune response in endotoxic shock. Therefore, the aim of this study was to investigate the effects of a maternal diet supplemented with TRP in 15-day-old breeding rats – at a critical age for the development of neural cells and immune function–, which are essential for host defenses against endotoxic shock. LPS was administrated by intraperitoneal injection (IP) and MLT was also administered (IP), in order to eliminate free radicals and to have a parameter for comparison. The target organ used in this study was the lung, since it shows high expression and activity of IDO [24,25], an enzyme with important antioxidant activity that is expressed in the presence of TRP.

2. Materials and methods

2.1. Reagents

The MLT, lipopolysaccharide (LPS), and L-TRP were obtained from SIGMA Chemical Co. (St. Louis MO, USA). The cytokine IFN-γ Enzyme-linked-immunoadsorbent-assay (ELISA) kits were purchased from R&D Systems Co. (Minneapolis, MN. USA). The lipoperoxidation assay was obtained from Oxford Biomedical Co. USA, and the nitric oxide and glutathione peroxidase activity kits were purchased from Calbiochem Co. USA.

2.2. Diet components

The ingredients used in the diet preparation were: Purina rodent chow (Purina Meals, St. Louis MO); vitamin, mineral alphacell, glucose, sacarose and dextrin mixtures obtained from ICN (Costa Mesa, CA), and vegetable oil obtained from a commercial source. The salt and solvents used were obtained from Merck (Mexico) and Sigma (St. Louis MO) (Table 1).

Table 1
Composition of the diets (gr/100 gr diet).

Components	Diet containing 23% protein
Pellet Chow	98
Corn meal	–
Dextrose	–
Sacarose	–
Dextrin	–
Vegetable oil	2.0
Mineral mixture Rh[a]	–
Vitamin mixture	–
Fiber cellulose	–
Protein percentage	23

2.3. Animal preparation and experimental design

Fifteen adult, female, Sprague-Dawley rats (200–250 g) were used in this study. They were housed individually in Plexiglas™ cages for diet quantification, where food consumption was recorded every second day throughout the study for all groups. The animal rooms were windowless with automatic temperature (22 ± 2 °C) and lighting control (12 × 12 h light-dark cycle). The rats received water *ad libitum* and a modified diet with a TRP concentration of 0.5 mg/100 g protein, which corresponds to double the daily requirement of this amino acid in rat diet. Control animals received standard laboratory chow (Purina). The rats were fed a diet of 23% protein over two weeks after mating. For the experimental phase in a normoproteic model, 100 15-day-old breeding rats with an average weight of 18–20 g were obtained from our *CIBO-IMSS* animal facility. All experiments were performed in duplicate. The animals were then divided into six groups (control-saline, LPS, MLT, TRP, LPS + MLT, and LPS + TRP). LPS, MLT and diluents were administered by IP. The control group received diluent only. The groups LPS, LPS + MLT, and LPS + TRP were injected with LPS (20 mg/kg, LD100 doses in a single injection at 12:00 h): the groups MLT and LPS + MLT were injected with MLT (10 mg/kg, ip, 30 min. before LPS administration). Animals were treated in accordance with the official technical specifications for the production, use and care of laboratory animals in Mexico (*"NORMA Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio"*).

2.4. Lipid peroxidation assay

The products of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA) were measured in lung tissue. Tissues were homogenized in ice-cold 20 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) with a polytron like stirrer, to produce 1:10 homogenates. Homogenates were centrifuged at 3,000 g for 30 min at 4 °C. The supernatant was collected and immediately assayed for products of lipid peroxidation (MDA and 4-HDA). An LPO kit was used for these measurements, as previously described (Oxford Biomedical Res. Inc.). The kit is advantageous because it has a chromogenic reagent that reacts with the lipid peroxidation products MDA and 4-hydroxyalkenals (4-OHA) at 45 °C, yielding a stable chromophore with maximal absorbance at a wavelength of 586 nM: the light wavelength and the low incubation temperature used for the measurements, eliminated interference and undesirable artifacts. Proteins were measured by the Bradford method using bovine serum albumin as a standard [26]. Results are expressed as nmol MDA + 4-OHA (mg protein).

2.5. Glutathione peroxidase activity

A modification of the Jaskot method [27] was used to measure glutathione peroxidase activity. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C, the supernatant was decanted and recentrifuged at 100,000 × *g* for 60 min at 4 °C and then, was diluted 1:2 with 50 μmol/L of potassium chloride (pH 7.6). The samples were stored at −80 °C. After the first centrifugation, a fraction of the supernatant (10 μL) was taken for protein analysis. Glutathione peroxidase (GPx) was measured indirectly, in 50 μL of the prepared homogenate, by a coupled reaction with glutathione reductase using cumene hydroperoxide activity, a spectrophotometric system modified for esophageal tissues. This activity was referred to as micromoles of oxidized NADPH/min/mg of protein (oxidized NADPH μM min/mg protein).

2.6. Nitric oxide (nitrate and nitrite) determination

Nitric oxide was measured indirectly (the metabolism from nitrites and nitrates), using a commercial package (Calbiochem Nitric Oxide Assay Kit, colorimetric 482650) that uses the nitrate reductase enzyme to convert the nitrate to nitrite, and then through the Griess reagent in order to quantify nitrates.

2.7. Determination of Interferon-γ (ELISA test)

To determine the IFN-γ concentration, the sandwich-type ELISA method was used. The test is designed to trace antigens: the antigen binds to a specific antibody that is immobilized in a solid support, and the complex antigen-antibody formed is detected by colorimetric reaction.

2.8. Statistical analysis

Data were analyzed using a variance test (ANOVA), and when the F values were significant, the Student-Newman-Keuls test was used to compare between groups. The accepted level of significance was $p < 0.05$.

3. Results

3.1. Levels of lipoperoxide metabolites

LPS induced an important two-fold increase in the lipid peroxidation levels in rats' lungs, as demonstrated in MDA and 4-HDA concentrations, when compared to the control group (Fig. 1); however, the groups that received MLT orTRP alone did not show significant changes in the lipoperoxidation metabolites, compared to the control group (Fig. 1). Furthermore, MLT and TRP were able to inhibit lipoperoxidation in the conditions of endotoxic shock that were induced by LPS (Fig. 1).

3.2. Glutathione peroxidase activity

There was a significant increase in glutathione peroxidase (Gpx) activity in the lungs of rats under endotoxic shock in the experimental group compared to the control group. Also, there was an increase in Gpx activity in the MLT group compared to the CTRL group, whereas, the Gpx activity was not significant in the TRP group when compared to controls (Fig. 2). However, MLT or TRP did not reduce the Gpx activity in the lungs of rats after the LPS effect (Fig. 2).

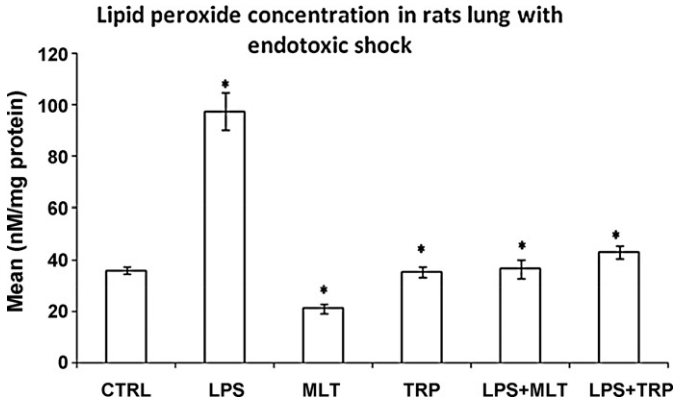

Fig. 1. Lipid peroxidation (MDA and 4-HDA) concentration in lungs of rats treated with lipopolysaccharide in six different groups: (CTRL, LPS, MLT, TRP, LPS plus MLT and LPS plus TRP). Lipid peroxidation was measured in nM/mg of protein. $^{*}p \leq 0.005$; CTRL vs LPS, LPS vs MLT, TRP, LPS + MLT and LPS + TRP groups. Data are expressed as mean ± SE.

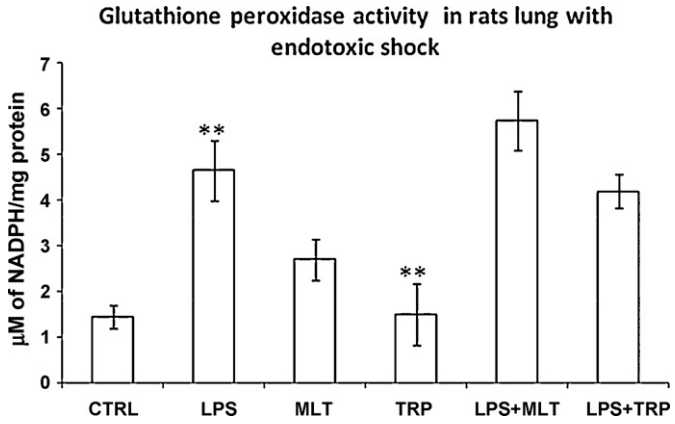

Fig. 2. Glutathione peroxidase activity in lungs of rats with endotoxic shock in six different groups: (CTRL, LPS, MLT, TRP, LPS plus MLT and LPS plus TRP). Activity of GPX was measured in μM of NADPH/min/mg of protein. $^{**}p \leq 0.01$; CTRL vs LPS. LPS vs MLT, TRP, LPS + MLT and LPS + TRP groups. Data are expressed as mean ± SE.

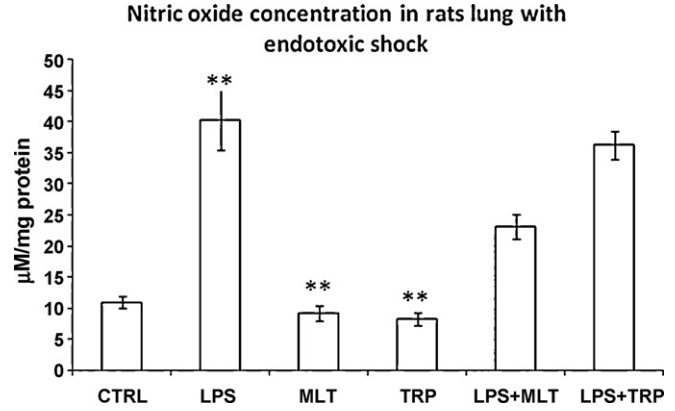

Fig. 3. Nitric oxide levels in lungs of rats with endotoxic shock in six different groups: (CTRL, LPS, MLT, TRP, LPS plus MLT and LPS plus TRP). Nitric oxide levels were measured in μM/mg of protein. $^{**}p \leq 0.01$; CTRL vs LPS. LPS vs MLT, TRP, LPS + MLT and LPS + TRP groups. Data are expressed as mean ± SE.

3.3. Nitric oxide levels

Levels of Nitric oxide (NO) were measured in the lungs of rats with and without the induction of endotoxic shock with LPS. Results showed a significant four-fold increase in the NO levels induced by LPS compared to the control group (Fig. 3). MLT and TRP alone did not modify NO levels but MLT significantly reduced the LPS effect on NO levels by 40%. TRP was unable to modify the LPS effect on NO levels (Fig. 3).

3.4. Serum interferon-gamma concentration

To determine the immune system response to the endotoxic shock induced by LPS, IFN-γ levels were measured in serum samples of the rats under the effects of LPS. Results showed an important increase in the IFN-γ levels induced by LPS (Fig. 4). However, MLT completely inhibited the LPS effect on IFN-γ levels, since those levels were similar to the control group (Fig. 4). In addition, TRP did not modify the IFN-γ concentration under the LPS effect, while MLT and TRP alone did not have any affect on IFN-γ levels (Fig. 4).

4. Discussion

Because of its antioxidant activity, TRP can be employed in the treatment of illnesses where MLT cannot, such as in autoimmune

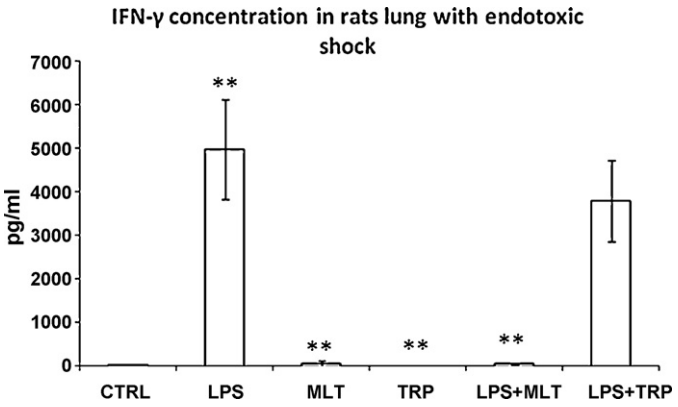

Fig. 4. Serum levels of interferon-γ in rats with endotoxic shock in six different groups: (CTRL, LPS, MLT, TRP, LPS plus MLT and LPS plus TRP). INF-γ levels were measured in pg/ml. ** $p \leq 0.01$; CTRL vs LPS. LPS vs MLT, TRP, LPS + MLT and LPS + TRP groups. Data are expressed as mean ± SE.

diseases like lupus erythematosus and multiple sclerosis, among others. This study informs about the antioxidant activity of TRP by evaluating its effects on 15-day-old breeding rats under endotoxic shock. This opens up a wide range of therapeutic possibilities for endotoxic shock, since the generation of lipoperoxides is an important factor in the process of cell death, and TRP represents an economical, innocuous option for the patient. There is an important parallel between nutritional state and immunity, involving interactions, synergisms, and antagonists because food quality can influence the host's nutritional state and affect its immunocompetence and resistance to illnesses.

Infectious processes that might affect growth, metabolism and specific nutritional needs directly affect immune system responses; and so, it is important to study the components of food that can contribute to regulating immune function: a study area that has been denominated as "immune-nutrition" and is characterized by the use and application of functional foods with the purpose of improving the system's immunological responses.

The aggression induced by a loss or unbalance of nutrients can directly affect the host's defense mechanism, reducing the organism's capacity for defense and favoring metabolic alterations [18,19].

Undernourishment and malnutrition impose significant risks in the incidence and mortality linked to infectious diseases, such as those provoked by gram-negative bacteria [19]. The LPS component of the membrane in gram-negative bacteria, like *Escherichia coli,* is capable of inducing oxidative damage in organs and systems through the generation of reactive oxygen species (ROS) that destabilize and cause loss of control in immune responses [20]. In our laboratory we worked with an endotoxic shock model and used MLT, a free radicals scavenger, to evaluate the efficiency of TRP and compare it with MLT. Under conditions of endotoxic shock, we approached the use of functional food with the amino acid TRP, which is capable of inducing the expression of antioxidant enzymes such as the synthesis of indolamine 2,3-dioxygenase (IDO) and endogenous MLT [28].

Additionally, besides being widely distributed in nature, MLT has been described as a stable amino acid molecule that is easily added to food and appears to have important adverse effects on some immune diseases [29].

Results of this study show that MLT and TRP are effective free radical scavengers (Fig. 1). According to the hypothesis proposed here, rats under endotoxic shock treated with TRP (Fig. 1) had lower lipoperoxide concentrations, but in the same group the changes in glutathione peroxide enzyme (GPx) activity were not significant (Fig. 2).

Recently, Feksa et al. [30] reported that high TRP concentrations induce "hypertryptophanemia." Individuals with this congenital disease, likely caused by a blockage in the conversion from TRP to kynurenine, accumulate TRP and some of its metabolites in plasma and tissues [29] in hypertryptophenamia it is possible to find oxidative stress and an imbalance between free radicals and the oxidative systems of the organism. The excessive accumulation of TRP (hypertryptophanemia) did not affect activity of the GPx enzyme, which is consistent with our results (Fig. 2).

Feksa et al. [30] suggested that the administration of TRP induces the generation of free radicals, among which are included the superoxide radical (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•). During this process, a significant increase in glutathione (GSH) levels is observed, probably due to oxidative stress through an over-regulation process, protecting the cells against damage by free radicals [28,30].

Glutathione is one of the most important antioxidative, nonenzymatic systems in the organism capable of reducing peroxides through the activity of GPx and free radical scavenging, besides regenerating oxidated vitamin C [28].

Contrary to Feksa et al. [30], who administered large quantities of exogenous TRP (2 μmol/g of weight) to rats of different ages to release ROS, and induced a TRP concentration about 10 times the normal plasma value in rats with the purpose of developing a disease similar to congenital hypertryptophanemia. In this work, we induced experimental endotoxic shock in healthy animals with a TRP concentration that corresponds to the required nutritional standard in humans (0.5 pg/g of protein). It would seem that the antioxidative effects of TRP could be predominantly directed by GSH but we did not observe significant activity of GPx in our experimental animals; although, we should not reject the possibility of using a higher TRP concentration and reevaluating GPx activity. In our model, and with the dietary administration of TRP in the concentration used (0.5 mg/100 g of protein), differences in nitric oxide concentration (NO) in rats under endotoxic shock (LPS group) compared to the rats under endotoxic shock that received TRP and MLT, respectively (Fig. 3), were not observed. Cubero et al. [26] demonstrated that the phagocytic activity and circulating levels of MLT were increased in animals that received a single dose administration of L-TRP at 125 mg/kg of weight. In a higher concentration of L-TRP (300 mg/kg), the circulating concentration of MLT was increased and the oxidative metabolism of the phagocytes was considerably improved [31]. On the other hand, *in vivo* studies indicate that the NO produced by the iNOS enzyme can inhibit the IDO enzyme's activity, promoting TRP degradation through the proteosome way [31–33]. These authors suggest that the NO-dependent oxidative regulation can be modulated by cellular factors as the abundance of NO, pH, the Redox environment, and TRP availability [32]. While, in this particular experiment, endotoxic shock model differences between NO concentration were not observed, it is very probable that the MLT concentration used in this case, would not be high enough to modify the NO levels in animals subjected to endotoxic shock and treated with MLT and TRP respectively (Fig. 3). It is important to note that MLT overthrew almost 99% of the circulating levels of the cytokine IFN-γ; while in rats under endotoxic shock that received the amino acid TRP, the differences were not significant compared to the LPS group (Fig. 4). Recent reports demonstrate that IFN-γ is able to induce the expression of IDO, the enzyme that catalyzes TRP [24,33]. We know that in endotoxic shock the levels of this proinflammatory cytokine are elevated and that the IDO expression could be induced by IFN-γ produced by Th1 activated cells in the central nervous system [34,35]. It is likely that in our model different situations may have occurred: we may not have used the necessary concentration of TRP, or perhaps the organism was using this amino acid to produce serotonin and MLT in important

quantities, in trying to counteract the effects of endotoxic shock (Fig. 4).

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