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**ORIGINAL ARTICLE** 

## THE NEUROPROTECTIVE EFFECT OF ERYTHROPOIETIN IN RAT HIPPOCAMPUS IN AN ENDOTOXIC SHOCK MODEL

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## ABSTRACT

**Background:** Sepsis is characterized by an early systemic inflammation in response to infection. In the brain, inflammation is associated with expression of pro-inflammatory cytokines (e.g. tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  and interleukin-6, among others) that may induce an overproduction of reactive oxygen and nitrogen species. The constitutive expression of cytokines in the brain is low, but may be induced by various stimuli, including lipopolysaccharide, which causes neuronal damage. Erythropoietin, among other effects, acts as a multifunctional neurotrophic factor implicated in neurogenesis, angiogenesis, vascular permeability, and immune regulation in the central nervous system. In an experimental model of endotoxic shock, we studied the neuroprotective capacity of erythropoietin in the rat hippocampus and compared with melatonin, a neurohormone with an important antioxidant and immunomodulatory effect. **Methods**: In 21-day-old male Wistar rats divided into eight groups, we administered by intraperitoneal injection lipopolysaccharide, erythropoietin, melatonin, or combinations thereof. The hippocampus was dissected and morphological (histological analysis) and biochemical (cytokine levels) studies were conducted. **Results:** The number of dead neuronal cells in histological sections in groups treated with lipopolysaccharide was higher compared to the erythropoietin group. There was a greater decrease (70%) in interleukin-1 $\beta$  concentrations in rats with endotoxic shock that received erythropoietin compared to the lipopolysaccharide group. **Conclusions:** The neuronal cell loss caused by endotoxic shock and interleukin-1 $\beta$  levels were reduced by the administration of the hematopoietic cytokine erythropoietin in this experimental model. (REV INVES CLIN. 2016;68:292-8)

Key words: Erythropoietin. Endotoxic shock. Neuroimmunology. Neuroinflammation. Oxidative stress.

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## INTRODUCTION

Endotoxic shock and sepsis represent an important clinical challenge worldwide<sup>1</sup>. Sepsis is characterized by early systemic inflammation in response to an infection and is associated with hypoperfusion, followed by tissue injury and subsequent organ damage<sup>1-3</sup>. The central nervous system (CNS) plays an important role in the production of cytokines and other immune factors<sup>3</sup>. In the brain, inflammation is associated with glial cell activation and proliferation, usually following an acute inflammatory response. Indeed, while the constitutive expression of cytokines in the brain is low, it can be induced by various stimuli, including lipopolysaccharides (LPS)<sup>4,5</sup>.

In the CNS, LPS induces the migration of activated lymphocytes and other immune cells, which cross the blood-brain barrier (BBB) or blood-cerebrospinal fluid barrier<sup>6</sup>. The transmigration of primed cells induces the BBB endothelium to relax its tight junctions, allowing the passage of cells carried in the blood into the CNS<sup>6</sup>. This process activates neurons and glial cells to express pro-inflammatory cytokines (e.g. tumor necrosis factor alpha [TNF- $\alpha$ ] and interleukins IL-1 $\beta$  and IL-6, among others), stimulates clusters of differentiation (CDs) like CD200 (neurons) and CD200R (microglia), as well as promoting the expression of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1)<sup>6</sup>. Elevated concentrations of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 may induce an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), both of which can cause injury during development in susceptible areas of the CNS, including the cerebral cortex and hippocampus7.

Studies in rats treated with LPS show alterations in the BBB and morphological changes in the hippocampus associated with neuronal death<sup>8,9</sup>. The hippocampus, specifically the CA1 region, is more susceptible to damage than other areas in the brain. Some features of this region that make it more vulnerable compared to other areas of the hippocampus or other brain regions include: (i) the highest density of AMPA and NMDA receptors that makes it more susceptible to excitotoxic damage caused by glutamate released by glutamatergic projections from CA3<sup>10</sup>; (ii) the lower density of blood vessels and increased susceptibility of these to show BBB alterations caused by ischemia<sup>11</sup>; and (iii) the increased vulnerability of astrocytes in the CA1 region to the damage caused by free radicals in the mitochondria<sup>12</sup>.

Thus, it is important to define new therapies for patients with endotoxic shock that can modulate the inflammatory immune response of the brain at early stages of maturation and development, which are critical for memory and learning. Interestingly, it appears that the alterations elicited by endotoxic shock may be prevented by the hematopoietic cytokine erythropoietin (EPO), which is produced in the liver, kidney, heart, and brain<sup>13-17</sup>, and by melatonin (MLT), synthesized not only by the pineal gland but also in retina, gastrointestinal tract, thymus, and bone marrow, among others. The protective effects of MLT against sepsis are suggested to be due to its antioxidant immunomodulating and inhibitory actions against the production and activation of pro-inflammatory mediators<sup>18-20</sup>. Novel biological activities of EPO have recently been described, such as those of a multifunctional neurotrophic factor implicated in neurogenesis, angiogenesis, vascular permeability, and immune regulation in the CNS<sup>13,17,21</sup>. The EPO does not normally cross the BBB but, during an inflammatory process such as that induced by LPS, the barrier's permeability is altered and, as cytokine levels increase, these are able to cross the BBB<sup>17,22</sup>. The aim of the present study was to evaluate the neuroprotective capacity of EPO in the rat CNS (hippocampus) following endotoxic shock.

## MATERIALS AND METHODS

## **Experimental groups**

All experiments were performed in 21-day-old male Wistar rats maintained on a 12/12 hour light/dark cycle at an ambient temperature of  $22 \pm 2^{\circ}$ C, with food and water *ad libitum*. Experiments were carried out following the Mexican guidelines for handling laboratory animals (Norma Oficial Mexicana para el Manejo de Animales de Laboratorio, NOM-062-ZOO-1999).

Animals were divided into eight groups (n = 10 each): (i) sham (intact rats); (ii) LPS (lipopolysaccharide from *Escherichia coli* O111:B4 15 mg/kg<sup>-1</sup>; Sigma St. Louis, MO, USA); (iii) MLT (melatonin 10 mg/kg<sup>-1</sup>; Sigma St. Louis, MO, USA); (iv) EPO (erythropoietin Figure 1. Representative photomicrographs of the hippocampal CA1 area in 21-day-old rats: **A**: sham; **B**: lipopolysaccharide (LPS); **C**: melatonin (MLT); **D**: erythropoietin (EPO); **E**: LPS + EPO; **F**: LPS + MLT; **G**: MLT + EPO; **H**: LPS + MLT + EPO. Paraffin-embedded tissue sections (8 µm) stained with hematoxylin and eosin and examined under an optic microscope (40x magnification).



5,000 U/kg<sup>-1</sup>; PISA, Mexico); (v) LPS + EPO (LPS 15 mg/kg<sup>-1</sup> + EPO 5,000 U/kg<sup>-1</sup>); (vi) LPS + MLT (LPS 15 mg/kg<sup>-1</sup> + MLT 10 mg/kg<sup>-1</sup>); (vii) MLT + EPO (MLT 10 mg/kg<sup>-1</sup> + EPO 5,000 U/kg<sup>-1</sup>); and (viii) LPS + MLT + EPO (LPS 15 mg/kg<sup>-1</sup> + MLT 10 mg/kg<sup>-1</sup> + EPO 5,000 U/kg<sup>-1</sup>). All chemicals were administered by intraperitoneal injection.

We used a concentration of LPS (LD75) to generate clinical signs and symptoms of acute shock and assess the activity of EPO. The MLT was used as a control due to its immunomodulatory activity and to compare the results with those of EPO in the endotoxic shock model.

## Histological methods

To characterize the effect of LPS and the other treatments in the hippocampal neurons, half of the rats (n = 5) in each group (Sham, LPS, MLT, EPO, LPS + EPO, LPS + MLT, MLT + EPO, and LPS + MLT + EPO) were studied histologically. Rats were anesthetized with a lethal dose of sodium xylazine (5 mg kg<sup>-1</sup>) and ketamine (80 mg kg<sup>-1</sup>) administered by intramuscular injection, and were then perfused with 180 ml of 0.9% NaCl containing 10 Ul<sup>-1</sup> of heparin and 0.01% procaine at body temperature for about five minutes. Animals were then perfused for 10 minutes with 280 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), at a perfusion pressure of 140 cm  $H_2O^{23}$ . The perfused brains were embedded in paraffin and serial coronal sections (10 µM) including the dorsal hippocampus (CA1 area) were stained with hematoxylineosin to visualize the degree of cell damage (Fig. 1). Cells were counted in 4-5 fields per section from four sections taken from each of the five animals studied from each group. Cells were counted under a light microscope (Leica DME, 40x magnification) equipped with an analog photomicroscope system, using ImageJ software<sup>24</sup>. Neurons with a characteristic regular morphology, regular cell membrane outline, homogeneous cytoplasm, and well-defined nucleus were classified as normal cells (live neurons). Cells that had any fragmentation, shrinkage, basophilic cytoplasm, pyknotic nucleus, swelling, ghost form, or vacuolization were classified as damaged or morphologically abnormal cells (dead neurons)<sup>25</sup>. Results were expressed as the number of dead neurons (cell death).

### Analysis of inflammation markers

The remaining five animals within each group were used for cytokine determination. Rats were sacrificed by decapitation 12 hours after inoculation; it has been shown that between eight and 12 hours after administration of LPS, endotoxic shock ensues and proinflammatory cytokine levels increase. The hippocampus was dissected out and maintained at  $-20^{\circ}$ C. Tissue was homogenized in PBS containing a protease inhibitor cocktail (Calbiochem-Novabiochem, San Diego, CA) at 500 µl per 50 mg of tissue. Protein expression was evaluated using enzyme-linked immunosorbent assays (ELISA) to measure rat TNF- $\alpha$ , rat IL-1 $\beta$ /IL-1F2, and rat IL-6 (all ELISA kits were obtained from Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA).



Figure 2. Dose-response curve to calculate the lethal dose (LD)<sub>50</sub>, LD<sub>75</sub> and LD<sub>100</sub> of lipopolysaccharide (LPS).

Absorbance was measured at 450 nm and wavelength correction was performed to 540 or 570 nm, using a Bio-Rad Microplate Reader Model 550.

### Statistical analysis

Data are presented as means and standard error. Due to the non-parametric distribution of the data, as shown by the Kolmogorov-Smirnov test (p < 0.05), the differences between groups were assessed using Kruskal-Wallis and Mann-Whitney *U* tests with significance set at < 0.05. All statistical analyses were conducted with the Statistical Program for Social Sciences v22.0 (SPSS, Inc.; Chicago, ILL, USA).

## RESULTS

#### Lipopolysaccharide dose-response curve

The appropriate dose of LPS to be administered intraperitoneally was calculated in a dose-response curve, generated by administering different LPS concentrations (5, 10, 15, 20, or 25 mg/kg<sup>-1</sup>) to groups of eight rats each (Fig. 2). From these curves, the LD50 (10 mg/kg<sup>-1</sup>), LD75 (15 mg/kg<sup>-1</sup>), and LD100 (20 mg/kg<sup>-1</sup>) were calculated to induce endotoxic shock. Clinical symptoms and manifestations of endotoxic shock in rats included rough hair, profuse diarrhea, conjunctivitis, and photophobia. During the necropsy, we observed internal bleeding and petechiae in liver and lung. The final concentration of LPS used in the model was the LD75.

#### Neuronal death

When we analyzed cell death in the hippocampal CA1 area (Fig. 3), there was a mean ( $\bar{x}$ ) of 265.25 dead neurons in sections from the control group, which represented 59.97% of the cell loss per mm<sup>2</sup> observed in the experimental group that developed endotoxic shock (LPS group, p  $\leq$  0.05). In the MLT group ( $\bar{x} = 215.50$ ) there were significantly fewer live neurons compared to the LPS group ( $\bar{x} = 305.75$ ) and LPS + EPO group ( $\bar{x} = 266.00$ ) displayed 53.85 and 59.85% less neuronal death, respectively (p  $\leq$  0.05 compared to the LPS group).

In the experimental groups that received MLT alone or in combination (LPS + MLT, = 261.25; MLT + EPO, = 284.50; and MLT + LPS + EPO, = 259.00), the proportion of neuronal death was lower than in rats that received LPS alone, representing a reduction of 60.57, 57.06, and 60.91%, respectively ( $p \le 0.05$ ).

#### Interleukin- $\mathbf{1}\beta$ levels

The minimum detectable dose (MDD) of rat IL-1 $\beta$  is typically < 5 pg/ml<sup>-1</sup>. The mean concentration of IL-1 $\beta$  in the rats that developed endotoxic shock was 1,166 ± 339.4 pg/ml<sup>-1</sup>, whereas this cytokine was





undetectable in the sham and MLT groups, representing up to 100% lower levels of this cytokine compared to the LPS group ( $p \ge 0.0001$ ). In the rats that received erythropoietin (EPO group), we obtained a mean value of 94.3  $\pm$  31.2 pg/ml<sup>-1</sup> of IL-1 $\beta$ , which was 91.91% lower than in the LPS group ( $p \ge 0.0001$ ). In the rats that received LPS + EPO, the mean levels were =  $325.8 \pm 35.5 \text{ pg/ml}^{-1}$ , 72.07% lower than in those that received LPS alone  $(1,166.5 \pm 339.4 \text{ pg/m}^{-1})$  $p \ge 0.005$ ), whereas in the animals that were subjected to endotoxic shock and received MLT (LPS + MLT group), the mean IL-1 $\beta$  levels were 245 ± 56.0 pg/ml<sup>-1</sup>, 78.95% lower than the levels of rats that received LPS alone ( $p \ge 0.002$ ). Consistent with the earlier results, the levels of IL-1 $\!\beta$  in the rats that received MLT + EPO were =  $4.2 \pm 4.2 \text{ pg/ml}^{-1}$ , 99.63% lower than in the animals that received the endotoxic shock alone ( $p \ge 0.0001$ ). Finally, in the rats that received MLT + LPS + EPO, the mean IL-1 $\beta$  levels were 24.9 ± 15.5 pg/ml<sup>-1</sup>, 97.86% less than in the rats that received LPS alone (1,166 ± 339.4 pg/ml<sup>-1</sup>,  $p \ge 0.0001$ ; Fig. 4).

# Tumor necrosis factor- $\alpha$ and interleukin-6 levels

In contrast to IL-1 $\beta$ , TNF- $\alpha$  and IL-6 were below the detection limit in this model of endotoxic shock. The minimum detectable dose (MDD) of rat IL-6 ranges from 14 to 36 pg/ml<sup>-1</sup>, and the MDD of rat TNF- $\alpha$  is typically < 5 pg/ml<sup>-1</sup>.

### DISCUSSION

Oxygen deficiency in tissues results in the generation of EPO, an increase in the expression of the EPO receptor, EPO-R, in kidney and liver. During a severe hypoxia event, EPO can be produced in the brain, as well as cross the BBB into the systemic circulation to reach peripheral organs. Histological analysis of the hippocampal CA1 region suggests significant differences between the different treatments in this study. The LPS-induced endotoxic shock increased neuronal death (Fig. 1 and 3), as it can reveal the presence of "ghost forms" and the loss of pyramidal cells in this area (Fig. 1). An increase in neuronal death was evident in the LPS groups (Fig. 3). However, when the LPS was administered with EPO (LPS + EPO group), we observed a smaller number of dead cells in the CA1 region, compared with dead cells in the group with endotoxic shock (LPS group). The increase in survival of functional neurons when animals were subjected to endotoxic shock in the presence of EPO could reflect signaling pathways through which EPO prevents apoptotic neuronal death through kinases and anti-apoptotic genes<sup>26,27</sup>. In addition, EPO has the ability to regulate the levels of oxidative stress, which induce the generation of ROS, including superoxide, hydrogen peroxide, oxygen singlet, hydroxyl radical, nitric oxide and peroxynitrites. The EPO limited the generation of these radicals and the extent of cell damage, resulting in a lower rate of neuronal death (Fig. 1 and 3).





Erythropoietin operates at different levels within the CNS, limiting the production of ROS, modulating neurotransmission, preventing apoptosis, and reducing the inflammatory process<sup>28,29</sup>; because of these effects generated by EPO, it is possible to suggest that it has neuroprotective effects.

During endotoxic shock, a large number of cytokines and pro-inflammatory mediators are generated, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Pro-inflammatory cytokines may downregulate the expression of EPOmRNA, but in turn, they can increase the expression of EPO-R in astrocytes<sup>28</sup>. The EPO attenuates inflammation by reducing reactive astrocytosis and microglial activation, thereby inhibiting the recruitment of immune cells to damage areas<sup>21,28,30</sup>; in cultured cerebrovascular endothelial cells, EPO downregulates the levels of TNF-  $\alpha$  induced by the expression of IL-6, as well as the levels of IL-1 $\alpha$ , chemokine receptor type 4 (CXCR4), and IL-1<sub>β</sub>. The EPO directly inhibits the effects of interferon- $\alpha$ , and the alterations through cytotoxicity induced by LPS in oligodendrocytes that affect the integrity of white matter<sup>28</sup>. Furthermore, EPO influences the release of TNF- $\alpha$  and reduces its effects in Schwann cells<sup>28</sup>. In this study, TNF- $\alpha$  and IL-6 were not detected, while the mean levels of IL-1 $\beta$ induced by endotoxic shock (LPS group) were reduced by nearly 72% in the presence of EPO (LPS + EPO group; Fig. 4).

Neuronal activation of the EPO-R to prevent apoptosis induced by N-methyl-D-aspartate (NMDA) receptor or nitric oxide involves the crosstalk of signals between the Janus Kinase-2 (JAK-2) and nuclear factor kappa beta-light chain enhancer of activated-B cells (NFkB) second messenger pathways, the latter being activated by IL-1 $\beta^{28,29}$ . The protection provided by the exogenously administered EPO is associated with the prevention of the increase in IL-1 $\beta$  levels and the attenuation of leukocyte infiltration after hypoxia/ ischemia-reperfusion injury<sup>31</sup>. In a large variety of nervous system disease models where EPO fulfills a protective role, inflammation is a pathogenic component induced by cytokines and chemokines, and it is followed by leukocyte infiltration or enhanced glial activation<sup>31,32</sup>. The EPO neuroprotection is attributed to a delay in the production and release of pro-inflammatory cytokines by microglia and astrocytes, as well as a significant reduction in the influx of inflammatory cells in the brain parenchyma<sup>31</sup>.

We have provided evidence suggesting that endogenous administration of EPO prevents the neuronal death induced by endotoxic shock, as well as diminishes the levels of pro-inflammatory cytokine IL-1 $\beta$  in the rat CA1 region. However, the molecular mechanisms through which exogenous administration of EPO exerts these protective effects are still not fully understood and only some components of the proposed pathways have been identified.

Neuronal cell loss caused by endotoxic shock, as well as IL-1 $\beta$  levels, can be reduced by the administration of the hematopoietic cytokine EPO during the early

stages of shock. Accordingly, EPO may provide important benefits when used to treat sepsis and endotoxic shock.

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