# Lipopolysaccharide-based endotoxemia produce toxicity in peripheral organs and microglia migration in a dose-dependent manner in rat *substantia nigra*

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## Abstract

*The peripheral inflammatory stimulus could induce cell damage in peripheral organs and activate microglial cells in the brain. One such stimulus was given to adult male Wistar rats by injecting different concentrations of lipopolysaccharide (LPS; 50, 300, 500 μg/kg and 5 mg/kg i.p.). To verify the systemic effect of the LPS administration, the serum content of C-reactive protein (CRP), the variation of body weight and cellular changes in the spleen, liver and kidney were determined. Motor impairment was evaluated by rotarod and open field tests. Microglia activation and dopaminergic degeneration was confirmed by immunolabelling for CD11b/c (microglia) and tyrosine hydroxylase (TH), respectively. The cell counting was performed in substantia nigra pars compacta (SNpc), microglial activation was explored in SNpc, substantia nigra pars reticulata (SNpr), substantia nigra pars compacta dorsal (SNcd) and the ventral tegmental area (VTA). For the statistical analysis, one-way ANOVA followed by Tukey post hoc test (p ≤ 0.05) was used. On day 7 post intraperitoneal administration of LPS, cellular atrophy was detected in the liver, kidney and spleen at 5 mg/kg, without significant changes in CRP levels. Body weight loss and motor impairment was present only on day 1 post LPS administration. The dosage of 500 μg/kg and 5 mg/kg of LPS caused the loss of dopaminergic neurons (40%) in SNpc and microglia migration in a dose-dependent manner in SNcd, SNpc and SNpr. LPS-induced endotoxemia favours damage to the peripheral organs and microglial migration in a dose-dependent manner in rat substantia nigra.*

*Key words: inflammation, microglia, dopamine, neurons, motor impairment.*

## Introduction

In animal models, it has been widely determined that the intranigral injection of lipopolysaccharide (LPS) induces oxidative stress, proinflammatory cytokines activation and microgliosis leading to the progressive loss of dopaminergic neurons in *substantia nigra pars compacta* (SNpc), a brain nuclei that is highly susceptible to toxins which is a hotspot in Parkinson's disease (PD) progression [2,7-9,12].

Recently, the effect of LPS-intraperitoneal immunization has shown relevance in pre-clinical approaches. The studies in rats and mice give us evidence that the intraperitoneal injection of single doses of LPS favours microglial activation in the SNpc [3,22,26,29]. However, it is not known whether

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the single LPS-intraperitoneal immunization leads to cell damage in peripheral organs; induces microglial migration and dopaminergic degeneration in a dose-dependent manner in rat *substantia nigra*.

The main purpose of this study was to evaluate the systemic effects of LPS-intraperitoneal immunization on adult male Wistar rats by measuring C-reactive protein (CRP) serum levels, body weight, cellular changes in peripheral organs, motor impairment, microglia activation, and dopaminergic degeneration. The novelty of the study is that a single administration of LPS via intraperitoneal injection induces dopaminergic degeneration in SNpc, and microgliosis in a dose-dependent manner in rat SNcd, SNpc, and SNpr.

## Material and methods

### Ethics

All the experimental procedures were performed according to the International Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1999), ethical committee of Instituto de Ciencias de la Salud of Universidad Veracruzana (approval number ICS-2017-008), NOM-062-ZOO-1999 and NOM-087-ECOL-SSA1-2002 from Mexican legislation. The n was defined according to Russell 3R principle. The Rat Grimace Scale [28] was used as criteria to avoid animal suffering. All efforts were made to minimize animal discomfort during the entire study.

### Experimental groups

Thirty-five adult male Wistar rats (200-300 g; 2.5 months old) were included in the study. The rats were housed in Plexiglas animal house at room cages (n = 5 per cage) under a 12/12 h light/dark cycle (light on at 8:00 a.m.) at room temperature (RT) with water and food *ad libitum*.

The rats were randomly organized into 5 groups (n = 7 per group). The control group was injected intraperitoneally (i.p.) with 500 μl of 0.9% saline solution. The other groups were independently administered with 50, 300 and 500 μg/kg, and 5 mg/kg LPS (*E. coli*, serotype 0111: B4, Cat. No. L2630; Sigma Aldrich, USA). The body weight of all groups was monitored daily (7:00-10:00 a.m.). Day 0 corresponded to the day of intraperitoneal LPS injection, and the perfusion was performed on day 7.

### Behavioural test

To evaluate the motor behaviour, rotarod and open field tests were used according to the previously described protocols [18,25]. Both tests were performed on day −1 (prior to the intraperitoneal injection of LPS), day 1 and day 7 after the injection. In case of the open field, a pre-test was considered for recognition and space habituation; and in the case of rotarod, a training period of 5 days was performed before the test.

Open field. This test was used to detect motor alterations and hypo or hyperactivity in the experimental subjects. Briefly, the test consisted of placing the individuals for 5 min in a dark acrylic box (44 × 33 × 20 cm), with the base divided into 11 × 11 cm squares. The variable measured was the number of cross squares taken as crossing criteria, when the subject passes at least three quarters of his body from one frame to another.

Rotarod. This test helps to evaluate motor coordination. The rotarod was used in the acceleration mode (4 to 40 rpm) for 5 min each. The registered variable was the latency to the fall and was measured in seconds.

### Quantification of C-reactive protein

The animals were euthanized with overdose of sodium pentobarbital (120 mg/kg). Two millilitres of blood was collected by cardiac puncture prior to the perfusion and was centrifuged at 3000 rpm for 10 minutes for the extraction of the serum, which was subsequently frozen at −20°C until the moment of determination of CRP. The sensitivity of the determination kit was 0.1 ng/ml. The quantification of CRP was performed by a spectrophotometer (Dirui CS-T240, Dirui Industrial Co. Ltd. in USA) and reagents from Sekure Chemistry (Sekure Chemistry Diagnostics, UK) in Facultad de Medicina Veterinaria y Zootecnia from Universidad Autonoma de Mexico. As a positive control (C+) of CRP, serum of rats that received 5 mg/kg i.p. LPS and stereotaxic intranigral injection was used. The LPS was administered in the SNpc by stereotaxic procedures under anaesthesia (Ketamine/Xylazine; 10 mg/kg/ 8 mg/kg) contemplating the coordinates, antero-posterior +2.4 mm from the interaural midpoint; medio-lateral +1.8 mm from the intraparietal suture; dorso-ventral −6.9 mm from the dura mater [17]. The LPS was injected with a flow of 0.2 μl/min with a micropump (Stoelting; USA).

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## Preparation of tissue for histology procedures

Immediately, transcardial perfusion was performed with 0.1 M phosphate buffer (PBS, from 10X PBS stock containing 8.1 mM $Na_2HPO_4$, 1.2 mM $KH_2PO_4$, 138 mM NaCl, 2.7 mM KCl, pH 7.4) followed by 4% paraformaldehyde (PFA 4%).

## Tissue staining with hematoxylin and eosin

The organs (liver, kidney and spleen) were removed after perfusion. Once extracted, they were placed in 10% formalin solution for 5 days. For the histopathological evaluation, a longitudinal section of 1 $cm^3$ of each organ was embedded in the paraffin block. Coronal sections of tissue of 5 µm thickness were made using a microtome (Leica MR2125RT). All organs were stained under the hematoxylin-eosin technique over the slides pre-treated with poly-L-Lysine (Sigma Aldrich, Inc.). Briefly, the tissue was exposed to Gill Hematoxylin, rinsed with double distilled water, and immediately immersed in lithium carbonate (0.5%), the histological sections were subsequently stained with Eosin; it was then dehydrated with ethanol (80%, 96% and 100%), followed by xylol. Each slide was covered with histological mounting medium (entellan) after protecting with a coverslip.

## Brain tissue processing

Brains were collected, postfixed for 48 h with 4% PFA at 4°C and then immersed in 30% sucrose solution prepared in 0.1 M PBS. A total of 54 mesencephalon coronal slices of 35 µm thickness in coronal plane distributed in 6 batches were collected by a cryostat (Leica CM1520, Leica Inc., Germany). Each series correspond to a representative sample, around 17% of *substantia nigra* total. The collected *substantia nigra* (considering SNpc, SNcd, SNpr and VTA) corresponds to interaural coordinates (4.20 mm to 2.96 mm) [24] and this tissue was used in immunostaining procedures.

## Immunohistochemistry for tyrosine hydroxylase

The protocol has been described previously [17]. Briefly, midbrain mesencephalon sections were permeabilized with 0.1% PBS-Triton. The endogenous peroxidase was inactivated with a solution containing 3% hydrogen peroxide and 10% absolute metha-

nol in 0.1 M PBS. Non-specific sites were blocked with 10% horse serum and 1% hydrogen peroxide in 0.1 M PBS for 1 h at room temperature (RT; 28-30°C). Then, the sections were washed with 0.05% PBS-Triton and incubated during 24 h at 4°C with mouse monoclonal anti-tyrosine-hydroxylase (TH; 1 : 1000; Cat. No. T1299, Sigma-Aldrich, USA). At the end of this incubation period, the sections were incubated for 2 h at RT with anti-mouse IgG (H + L) biotinylated, made in horse (1 : 200; Cat. No. BA2000, Vector Laboratories, Inc. USA). Then it was incubated with the ABC Elite Standard kit (Cat. PK6100; Vector Laboratories Inc., USA) for 2 h at RT followed by diaminobenzidine staining (DAB; Cat. SK100, Vector Laboratories Inc., USA). The sections were mounted on slides previously treated with 0.1% poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA) and protected with coverslips and entellan.

## Immunofluorescence for tyrosine hydroxylase and CD11b/c

Tissue sections of 35 µm were permeabilized with PBS-Triton 0.1% for 10 min, and nonspecific sites were blocked for 60 min with 10% horse serum in 0.1% PBS-Triton. The sections were incubated for 18 hours at 4°C with polyclonal anti-TH made in rabbit (1 : 1000; Cat. No. 6211 Abcam Inc., USA) and monoclonal anti-CD11b/c (OX-42) produced in mouse (1 : 300; Cat. No. 1211 Abcam Inc., USA). The secondary antibodies used were Alexa Fluor 488 anti-mouse IgG (H + L) made in donkey (1 : 200, Cat. No. 150105, Abcam Inc., USA) and Texas Red anti-rabbit IgG (H + L) produced in goat (1 : 200; Cat. No. TI-1000, Vector Laboratories Inc., USA). At the end, the tissue was exposed to 1 µM Hoechst 33342 (Cat. B2261, Sigma Aldrich, USA). The sections were mounted on slides previously treated with 0.1% poly-L-lysine solution. The fluorescence was protected with mounting medium (Vectashield, Cat. No. H-1000, Vector Laboratories Inc., USA).

The sections stained with hematoxylin and eosin as well as the tissue immunostained for TH and microglia, were acquired with a Leica DM1000LED microscope coupled to a Leica DFC450C camera and Leica AF software (Leica Microsystems, Wetzlar, Germany).

## Cell counting

The entire SNpc was divided and identified at three levels basing on the coordinates 4.20 mm (rostral), 3.80 mm (medial) and 3.40 mm (caudal) by taking an interaural line as a reference [24]. Quantification of

TH+ cells was performed in three 40× magnification micrographs at each level of the bilateral SNpc for each experimental group (around 5.5% of the total *substantia nigra*). The dopaminergic neuronal cells were counted according to the next inclusion criteria: the cells that conserved at least 90% of their soma and that touch the lower or right frame provided by ImageJ software were counted [17].

## Statistical analysis

All the results were expressed as a mean ± standard error of mean (SEM). For cell counting, body weight and CRP content, one-way ANOVA was used. The values obtained in the behavioural tests were analysed by means of one-way ANOVA for repeated samples ($p \leq 0.05$). Each ANOVA was followed by Tukey *post hoc* test.

# Results

## Systemic effect of LPS-based endotoxemia

The systemic effect of LPS was evaluated by rat behavioural responses, body weight measurement, CRP quantification and cellular damage in organs.

In order to know if the LPS also affects body weight, a total of 7 days before and 7 days after the intraperitoneal injection of LPS was recorded (Fig. 1A). In the vehicle group, the rats gained weight gradually (around 4 g per day), however, the experimental groups showed a significant body weight loss on Day 1 [$F_{(4,28)}$ = 7.948, $p < 0.001$]. From day 2 after LPS injection until day 7, the tendency of body weight gain was gradually recovered (Fig. 1A).

The levels of CRP in serum did not show significant differences between the experimental groups [$F_{(4,10)}$ = 0.181, $p = 0.943$]. All groups showed statistical differences, $p \leq 0.05$ with respect to positive control (C+), as was expected (Fig. 1B).

As per the histopathological observations, in the groups of 50 and 300 µg/kg LPS injection, slight hepatic necrosis was observed, accompanied by an increase in size of the sinusoidal space and vasodilation, being more drastic in the group injected with 5 mg/kg (Fig. 1C). In terms of renal parenchyma, the 300, 500 µg/kg, and 5 mg/kg LPS concentrations lead to slight glomerular atrophy, with diffuse epithelial cell detachment within proximal tubules. The group of rats treated with 5 mg/kg LPS exhibits collapsed glomeruli and cellular hypertrophy, which is associ-

ated with severe renal damage. In the spleen, all the experimental groups, unlike the control, showed follicular hyperplasia and diffuse haemorrhage.

The behavioural test was recorded by two paradigms. In the open field test, on day 1 all the experimental groups showed a significant reduction in the number of crossed squares [Vehicle [$F_{(2,18)}$ = 0.947, $p = 0.406$]; 50 µg/kg [$F_{(2,18)}$ = 7.12, $p = 0.005$]; 300 µg/kg [$F_{(2,18)}$ = 8.688, $p = 0.002$]; 500 µg/kg [$F_{(2,18)}$ = 4.742, $p = 0.022$]; 5 mg/kg [$F_{(2,18)}$ = 10.085, $p = 0.003$]. However, 7 days after LPS administration, the variable crossed squares were similar to those in the vehicle group (Fig. 2A).

On day 1, the rotarod results showed a decrease in the variable latency to fall in the groups injected with 300 µg/kg [$F_{(2,12)}$ = 7. 729, $p = 0.007$], 500 µg/kg [$F_{(2,12)}$ = 29.995, $p < 0.001$] and 5 mg/kg [$F_{(2,8)}$ = 8.496, $p = 0.010$] (Fig. 2B). The reduction in latency in this test indicates that the rats show motor impairment.

## Progressive loss of the dopaminergic neuronal population and distinct spatial distribution of microglia

In the SNpc, the intraperitoneal administration of 500 µg/kg LPS resulted in a decrease of 45.7% of TH + neurons, while the dose of 5 mg/kg LPS induced a loss of 53% when compared with the vehicle group [$F_{(4,20)}$ = 9.472, $p < 0.001$]. The groups of 50 µg/kg and 300 µg/kg did not show any significant differences as compared to the vehicle group (Fig. 3A-B).

Furthermore, the peripheral immunization induces microglial migration in a dose-dependent manner mainly in SNcd, followed by SNpc and SNpr. No microgliosis was found in VTA (Fig. 3C). The rats which received 5 mg/kg of LPS were stained OX-42+ and showed completely branched microglia vs. vehicle group in their morphology (Fig. 3D).

# Discussion

## Systemic effect

Previous reports showed a decrease in food and water consumption in the first 24 h after LPS intraperitoneal injection in rats [2,19,30]. Even though our present study did not focus much on the levels of cytokines, some existing reports link the decrease in rat body weight with the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) [21]. TNF-α, produced mainly by monocytes,
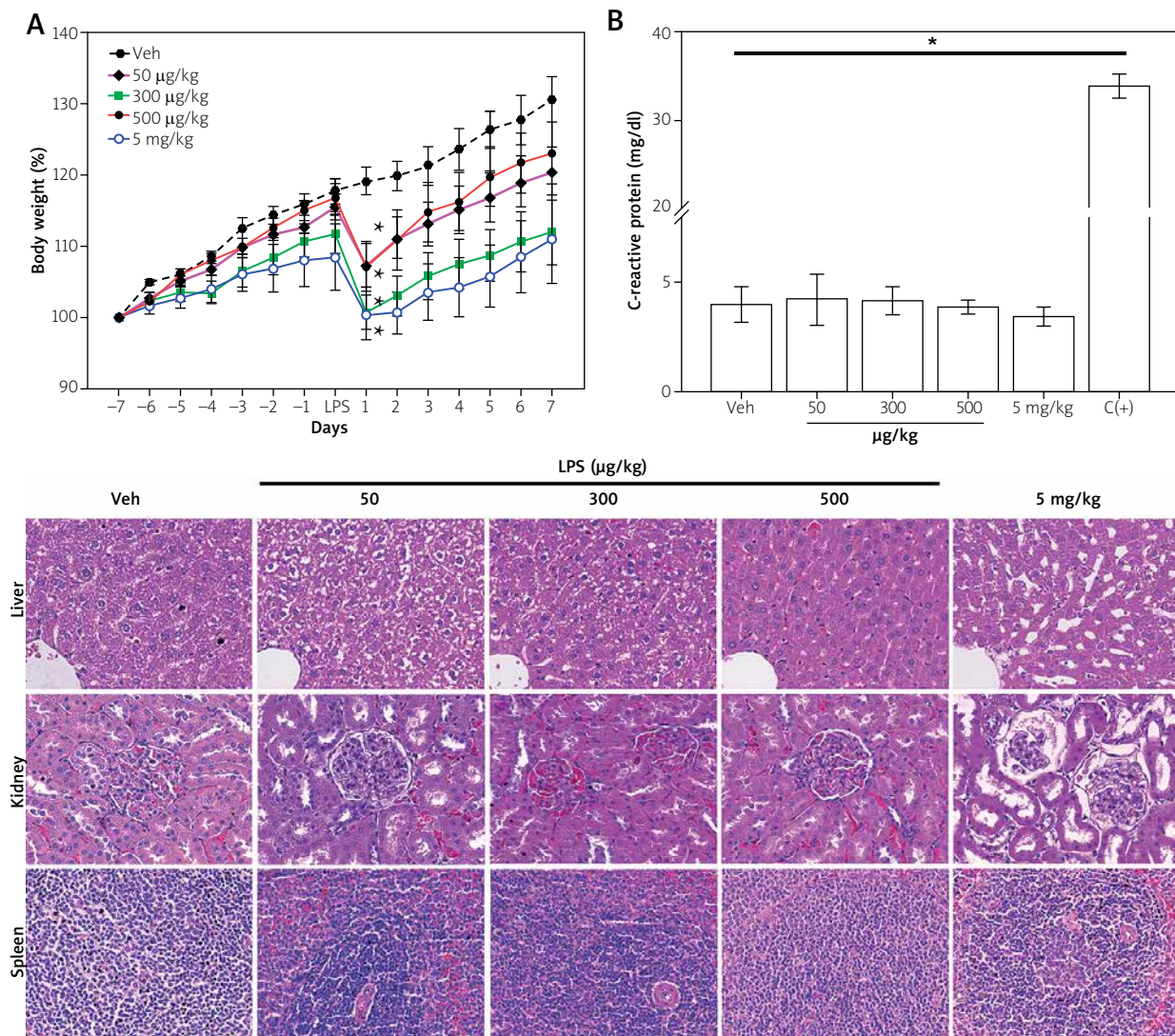
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**Fig. 1.** Systemic effects of LPS-intraperitoneal injection in rats. **A**) Body weight tendency per day. **B**) Levels of CRP in rat serum. C+ corresponds to double injection of LPS. *indicates the comparison of treatment vs. vehicle in graphic 1A, or the comparison of treatment vs. C(+) in plot 1B. All the results are expressed as a mean ± SEM. One-way ANOVA followed by Tukey *post hoc* test ($p \leq 0.05$). **C**) Representative micrographs of the liver, kidney and spleen stained with hematoxylin-eosin. Scale bar = 100 µm is the same for all the images.

macrophages and T lymphocytes, is a potent inducer of muscle metabolism and inhibitor of appetite (cachexia precursor), which can stimulate lipolysis by inhibition of lipoprotein lipase, the enzyme key to release fatty acids from circulating lipoproteins [31]. In addition, based on our observations, the loss of body weight could also be related to the decrease in motor activity and feeding disruption due to LPS-based visceral pain. With respect to motor impairment, a similar result of decrease in the locomotor activity at 24 h after the LPS intraperitoneal injection

in comparison with the controls was also reported by Godbout *et al*. [14]. This could attribute to sickness behaviour, which develops in sick individuals during the course of an infection or trauma, presenting with lethargy, decreased motor activity, depression, anxiety, loss of appetite and fever [10,11,20].

Another variable analysed was CRP content in serum. CRP is an acute phase protein of the inflammatory process expressed in response to non-specific stimuli like atherosclerosis, ischemia, necrosis, and vascular injury [15]. Its activation has been reported
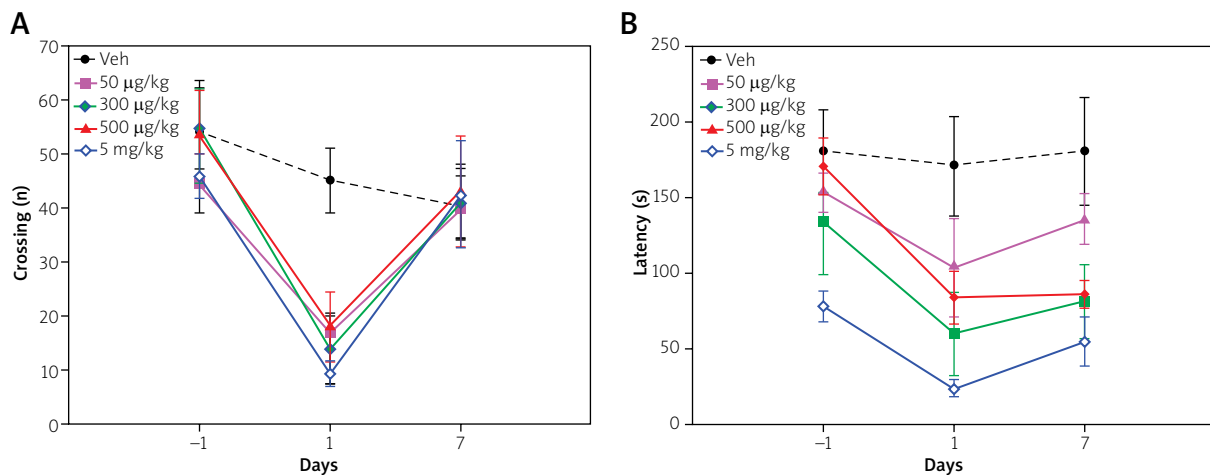
**Fig. 2.** Motor behaviour in Wistar rats. **A)** Number of crossed squares in an open field test. **B)** Latency to the fall in the rotarod. All the results are expressed as a mean ± SEM. One-way ANOVA for repeated samples followed by Tukey post hoc test ($p \leq 0.05$); *n* – number, s – seconds.

from 4 to 50 h post LPS injection [33]. Our results (3-4 mg/dl) are below the levels reported by other authors (10.87 ±0.13 mg/dl) [16]. Although we show basal levels in vehicle and C(+) groups, our data show that, regardless of the dose, no presence of inflammatory process exists 8 days after LPS administration.

On the other hand, the histological alterations observed in the organs studied suggest sepsis-type physiological alterations such as intraparenchymal damage in the lungs, heart, liver, spleen and kidney [5,6]. In hepatic parenchyma, we found mild hepatic necrosis, increase in the size of the sinusoidal space and vasodilation, as was reported by Abdel-Salam *et al.* [1]. They administered 300 µg/kg of LPS i.p. and analysed liver parenchyma 4 h after the injection; in this study, large areas of pericentral necrosis was identified with the loss of hepatic architecture, changes in vacuolar fat and infiltration of inflammatory cells. It has been described that at the beginning of an infectious disease, liver can reflect hyperbilirubinemia and renal failure can present around 10 to 14 days after the bacterial stimulus [23]. In our conditions, no cell infiltration or massive liver damage was obtained, which could explain the absence of significant changes in CRP levels 7 days post intraperitoneal LPS immunization.

With respect to kidneys, alterations due to LPS i.p. injection, we detected glomerular damage, which has already been reported, together with intrarenal hemodynamic changes, endothelial cell dysfunction and infiltration of inflammatory cells in acute kidney damage induced by sepsis [27]. In the case of the spleen, we confirmed the histological description published by Xiao *et al.* [32]. After LPS injection, they found alterations in the histological structure of the spleen when observing that the red pulp was intensely congested with red hemolyzed cells and the white pulp became very diffuse with a great decrease in the number of lymphocytes.

## Association of microglial activation and TH+ cell loss with LPS peripheral immunization

In our study, we found that a single dose of 500 µg/kg and 5 mg/kg of LPS causes a decrease in the dopaminergic population and microgliosis exacerbated 7 days after injection as per the hypothesis suggested by Block and Hong [4], who report that the dopaminergic degeneration mediated by inflammation is due to the oxidative stress produced by the reactive microglia.

The influence of microgliosis on neuronal damage has been reported using LPS-based endotoxemia. Cheng *et al.* [9] found that after the intraperitoneal injection of 5 mg/kg LPS, there exist changes in the hippocampal and cerebral cortex cellular morphology and microglia activation. In this context, Lin *et al.* [22] injected 300 µg/kg LPS i.p. in rat pups (PD21) and observed 12 hours later, an increase in the number of CD11b (+) cells, a microglia marker. Also, Sun *et al.* [29] determined that the intraperitoneal injection of LPS induced an increase in Iba-1 positive
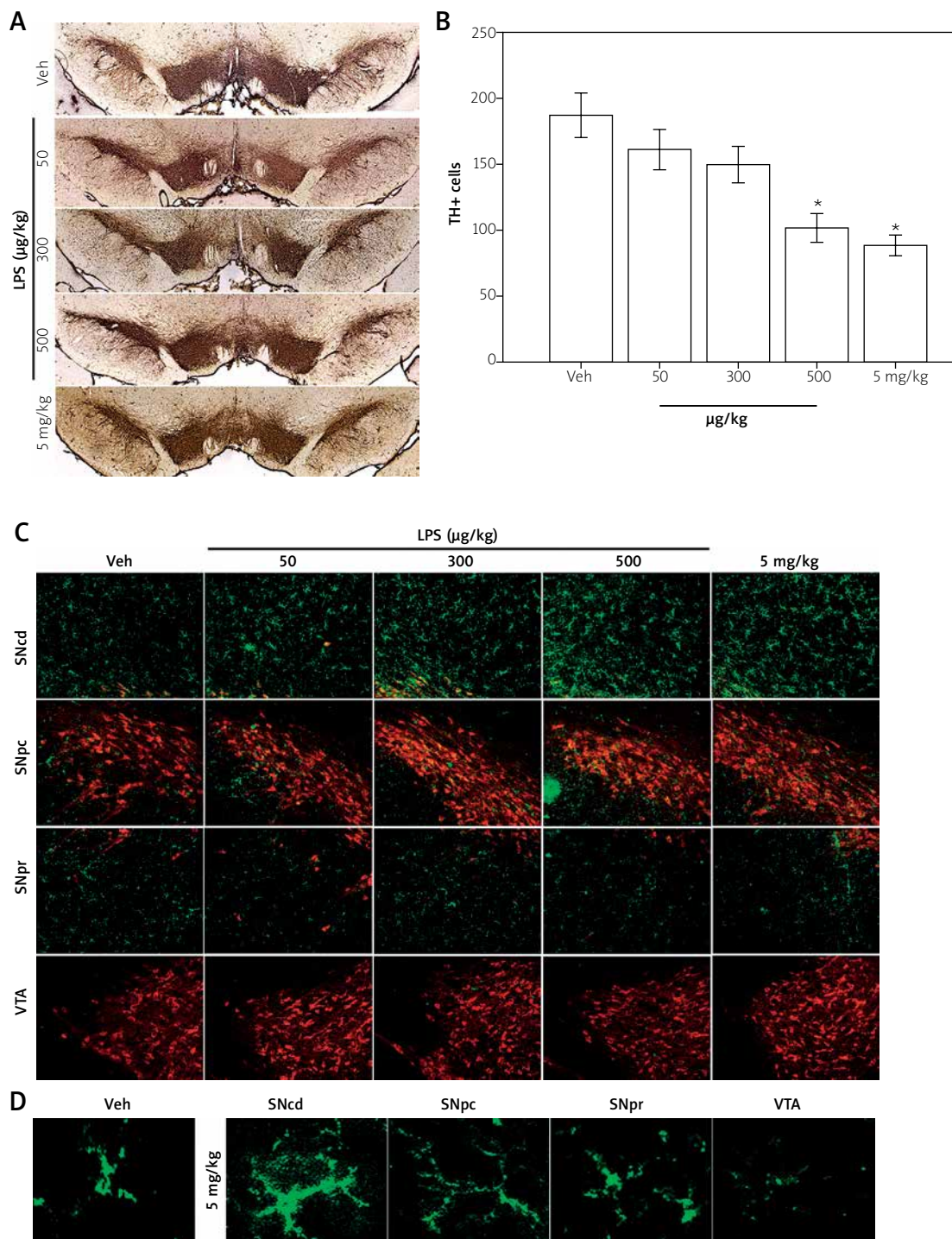
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**Fig. 3.** Intraperitoneal administration of LPS induces the loss of dopaminergic neurons and microgliosis in *substantia nigra*. **A**) Immunostaining for tyrosine hydroxylase (TH+). Scale bar = 200 μm. **B**) TH+ cell counting. All the results expressed as a mean ± SEM. One-way ANOVA followed by Tukey *post hoc* test $p < 0.001$. *Treatment vs. vehicle. **C**) Presence of microgliosis in SN and VTA. TH+ neurons (red channel), OX-42+ microglial cells (green channel) Scale bar = 100 μm. **D**) Morphological view of microglia posterior to LPS-based peripheral immunization. Scale bar = 5 μm. SNpc – *substantia nigra pars* compacta, SNcd – *substantia nigra compacta dorsal*, SNpr – *substantia nigra pars* reticulata, VTA – ventral tegmental area.

cells, also a microglia marker, observing changes in hippocampal microglia morphology such as enlargement of the cytoplasm and cell bodies, which is the characteristic feature of reactive microglia. Our data of microglial morphological changes are in accordance with a previous report of Flores-Martinez *et al.* [13]. For this, the accumulation of microglia (active) around of SNpc could exacerbate the damage to dopaminergic neurons, as has been observed in this study (loss of 40% of dopamine neurons) and that is in line at 47% referred by Qin *et al.* [26].

## Conclusions

The intraperitoneal injection of 500 μg/kg and 5 mg/kg of LPS produces liver and kidney damage without CRP level changes, transient body weight loss and motor impairment. The present paper showed that the lipopolysaccharide-based endotoxemia induced loss of TH+ neurons and microgliosis, in a dose-dependent manner in the SNcd and SNpc of adult male Wistar rats.

## Disclosure

The authors report no conflict of interest.

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