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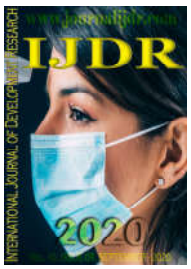

RESEARCH ARTICLE OPEN ACCESS

PROTECTIVE EFFECT OF *Lonchocarpus araripensis* LECTIN IN RAT POLYMICROBIAL SEPSIS

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ABSTRACT

Objective and design: To evaluate the effect of the seed lectin from *Lonchocarpus araripensis* (LAL) in inflammation, clotting and vascular dysfunction in septic rats. **Methods:** Sepsis was induced by cecal ligation and puncture (CLP) in male Wistar rats and evaluated 24 h later: 1. Peritoneal fluid - leukocyte migration, myeloperoxidase (MPO) activity and content of malondialdehyde (MDA), NO_2^-, interleukins (IL-1β, IL-6, IL-10); 2. Blood - coagulation tests (aPTT and PT) and platelet aggregation (ADP); 3. Aorta - vascular reactivity to phenylephrine (Phe) and acetylcholine (ACh). Animals were treated with LAL (1 mg/kg; i.v.) 30 min before sepsis induction. Results were analyzed by Student *t* test or ANOVA followed by Bonferroni test ($p < 0.05$) and expressed as Mean ± S.E.M. (n = 5-7). **Results:** LAL reduced total leukocytes (43%), neutrophils (39%), NO_2^- (100%), MDA (63%), MPO activity (70%), IL-1β (70%), IL-6 (78%) and the clotting time in the PT test (CLP: 35 ± 2.4 s *vs*. Sham 21.5 ± 3.5 s), and increased IL-10 (43%). LAL attenuated vascular dysfunction by the increase of ACh-induced relaxation (LAL: 0.00035 *vs*. CLP: 0.00016 AUC) and Phe-induced contractility (LAL: 0.0013 *vs*. CLP: 0.00056 AUC). **Conclusions**: LAL protects inflammation, bleeding and vascular dysfunction in rat polymicrobial sepsis.

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INTRODUCTION

Sepsis is a systemic inflammatory response to an infectious disease that triggers disordered release of inflammatory mediators, leukocyte migration, activation of platelet and clotting (ILAS, 2015; Gyawali, Ramakrishna, Dhamoon, 2019), being associated to 40% mortality. Sepsis leads to endothelial dysfunction and may result in the multiple organ dysfunction syndrome (Wort, Evans, 1999). Due to its varied clinical manifestations, the early recognition and diagnosis of sepsis may prevent the transition into septic shock. The role of animal endogenous lectins, such as galectins, modulating the production of inflammatory mediators, cell adhesion, apoptosis, chemotaxis and endocytosis in sepsis has been shown (Liu, Rabinovich, 2010; Ferreira *et al*., 2018; Kadowaki *et al*., 2013).

In this view, exogenous lectins could also interfere with the deregulated inflammatory process of sepsis. Plant lectins isolated from leguminous seeds inhibit vasoconstriction (Assreuy *et al*., 2009; Silva *et al*., 2012) and inflammation in models of paw edema and infectious or non-infectious peritonitis (Assreuy *et al*., 1997; Alencar *et al*., 2005; Napimoga *et al*., 2007), including those isolated from the genus *Lonchocarpus*, such as *L. sericeus* (Alencar *et al*., 2005; Napimoga *et al*., 2007), *L. campestris* (Pireset *al*., 2019a) *and L. araripensis,* focus of the present study (Amorim *et al*., 2016; Pires *et al*., 2016; Pires *et al*., 2019b; Pires *et al*., 2017). However, there are no studies referred to leguminous lectins in experimental models of sepsis. The present study aimed to evaluate the preventive effect of *Lonchocarpus araripensis*

lectin in inflammation, clotting and vascular dysfunction present in rat polymicrobial sepsis.

MATERIALS AND METHODS

Chemicals and reagents: Ketamine and xylazine were purchased from KÖNIG S.A., Avellaneda-BA, Argentine; adenosine diphosphate (ADP), phenylephrine (Phe), acetylcholine (ACh) and kits for dosage of nitrite and malondialdehyde (MDA) from Sigma-Aldrich Brazil Ltda, São Paulo, Brazil; brain thromboplastin from Trinity Biotech, Wicklow, Ireland; activated partial thromboplastin time (aPTT) and prothrombin time (PT) kits from Bios Diagnóstica (Sao Paulo, Brazil); ELISA kits from Biovision (California, USA). All other chemicals and reagents were of analytical grade.

Lectin: LAL, a N-acetyl-glucosamine-specific lectin, was isolated from seeds of *Lonchocarpus araripensis* Benth (family Leguminosae, tribe Dalbergieae; assessment code: AA73E7E at National System for the Management of the Genetic Heritage and the Associated Traditional Knowledge – SISGEN) by affinity chromatography in a chitin column followed by ion exchange chromatography (DEAE - Sephacel) (Pires *et al*., 2016). LAL was dissolved in sterile saline (NaCl 0.9%) immediately before use.

Animals: Male Wistar rats (250-300 g) were housed in controlled environment (circadian cycle, 20±2ºC), receiving food and water *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of Ceará (CEUA/UECE Nº. 46808261/2015).

Sepsis Induction: Rats were submitted to cecal ligation and puncture (CLP) (Baker *et al*., 1983) after intraperitoneal (i.p.) anesthesia with ketamine (90 mg/kg) and xylazine (10 mg/kg). CLP group had the cecum exposed, ligated, perforated ten times (sterile needle 18-gauge), pressed for extravasation of fecal content and both musculature and skin were sutured (sterile 4-0 silk line). Control group (Sham) underwent similar laparotomy and cecal exposure. All animals received intravenous (i.v.) injection of LAL (1 mg/kg) (Pires *et al*., 2016) or saline 30 min before surgery. Twenty-four hours after sepsis induction, blood was collected for coagulation (aPTT and PT) and platelet aggregation tests. The peritoneal fluid was collected for leukocyte count and dosage of inflammatory mediators, and aorta isolated for the vascular reactivity assays.

Inflammatory parameters: Total and differential leukocytes were counted by optical microscopy (Souza, Ferreira, 1988). Cytokine levels (IL-6, IL-1β and IL-10) were determined using ELISA kits; oxidative stress was estimated by the measurement of myeloperoxidase activity (MPO) (Bradley, Christensen, Rothstein, 1982), and content of malondialdehyde (MDA) (Huong*et al*., 1998) and nitrite (NO_2^-) (Green *et al*., 1982).

Aorta reactivity: Segments of thoracic aorta (3-5 mm) were removed and mounted for tension recording (2 g) in 10 ml organ baths filled with Tyrode's solution (in mM: 136 NaCl, 5 KCl, 0.98 $MgCl_2$, 2 $CaCl_2$, 0.36 NaH_2PO_4, 11.9 $NaHCO_3$, and 5.5 glucose) at 37 ºC, 95% O_2 and 5% CO_2, pH 7.4. Tissues were maintained during 1 h for equilibrium before being challenged with KCl (60 mM) in order to assure viability. The contractile response was measured using a force transducer coupled to a pre-amplifier and computerized data acquisition system (PanLab-DATAQ). For assessment of endothelium-dependent relaxation, ACh (10^{-8} - 10^{-5} M) was added to tissues pre-contracted with Phe (0.1 µM). The contractile response was evaluated by cumulative addition of Phe (10^{-8} - 10^{-5} M) (Araújo *et al*., 2011).

Hemostasis parameters: The animals blood samples were collected in tubes containing 3.2% sodium citrate and centrifuged for obtaining platelet-rich plasma (PRP, 118 × g, 10 min, 25 °C) and platelet-poor plasma (PPP, 1062 × g, 15 min, 25 °C). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured in coagulometer (CLOTimer DRAKE, Brazil), according to the instructions of manufacturer, with citrated plasma from CLP or Sham rats. In the aPTT test, 100 µl of plasma was incubated with 100 µl cephalin (37 ºC, 3 min) and the reaction was initiated by addition of 0.25 M $CaCl_2$ (100 µl). In the PT test, 200 µl thromboplastin was incubated at 37 °C for 5 min and plasma was added to initiate the reaction. In both tests, clotting time was measured up to 300 s and the assays were performed in triplicate. Platelet rich plasma (PRP; 450 µl) was warmed at 37 ºC in aggregometer cuvettes (Qualiterm PA.04, Brazil). Platelet aggregation was registered for 5 min in presence or absence of the agonist ADP (3 µM). The aggregation was quantified as the maximal light transmittance in relation to ADP (100% T) (Born, Cross, 1963).

Statistical analysis: Data was expressed as Mean ± S.E.M (n = 5-7/group) and analysed by Student *t* test or ANOVA followed by Bonferroni's test. Values of $p<0.05$ were considered significant.

RESULTS

LAL reduces leukocyte migration to peritoneal cavity of septic rats: CLP increased by 83% total leukocytes (22.4 ± 1.28 x 10^6 cells/mL *vs*. Sham 4.4 ± 0.32 x 10^6 cells/mL) and by 91% neutrophils (20 ± 1.31x 10^6 cells/mL *vs.* Sham 1.61± 0.1 x 10^6 cells/mL). LAL reduced both total leukocytes by 43% (15.3 ± 1.6 x 10^6 cells/mL) and neutrophils by 39% (11.9 ± 0.932 x 10^6 cells/mL) (Figure 1).

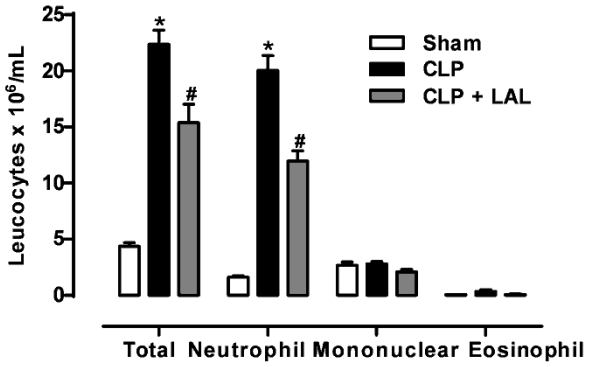

Fig. 1 LAL reduces leukocyte migration of septic rats. CLP or Sham animals received LAL (1 mg/kg; i.v.) 30 min before surgery and leukocyte migration to peritoneal fluid was determined 24 h thereafter. Mean ± SEM (n = 5-7). *p<0.05 vs. Sham; #p<0.05 vs. CLP.

LAL modulates oxidative stress and cytokine release in peritoneal fluid of septic rats: Sepsis induced by CLP increased the levels of NO^{2-} in 4.8x (4.1 ± 1.2 *vs.* Sham: 0.8 ± 1.6 µM), MDA in 27x (194.6 ± 39.3 *vs*. Sham: 7.09 ± 7.6 µmol/mL) and the activity of MPO in 16.5x (121.8 ± 23 *vs*.

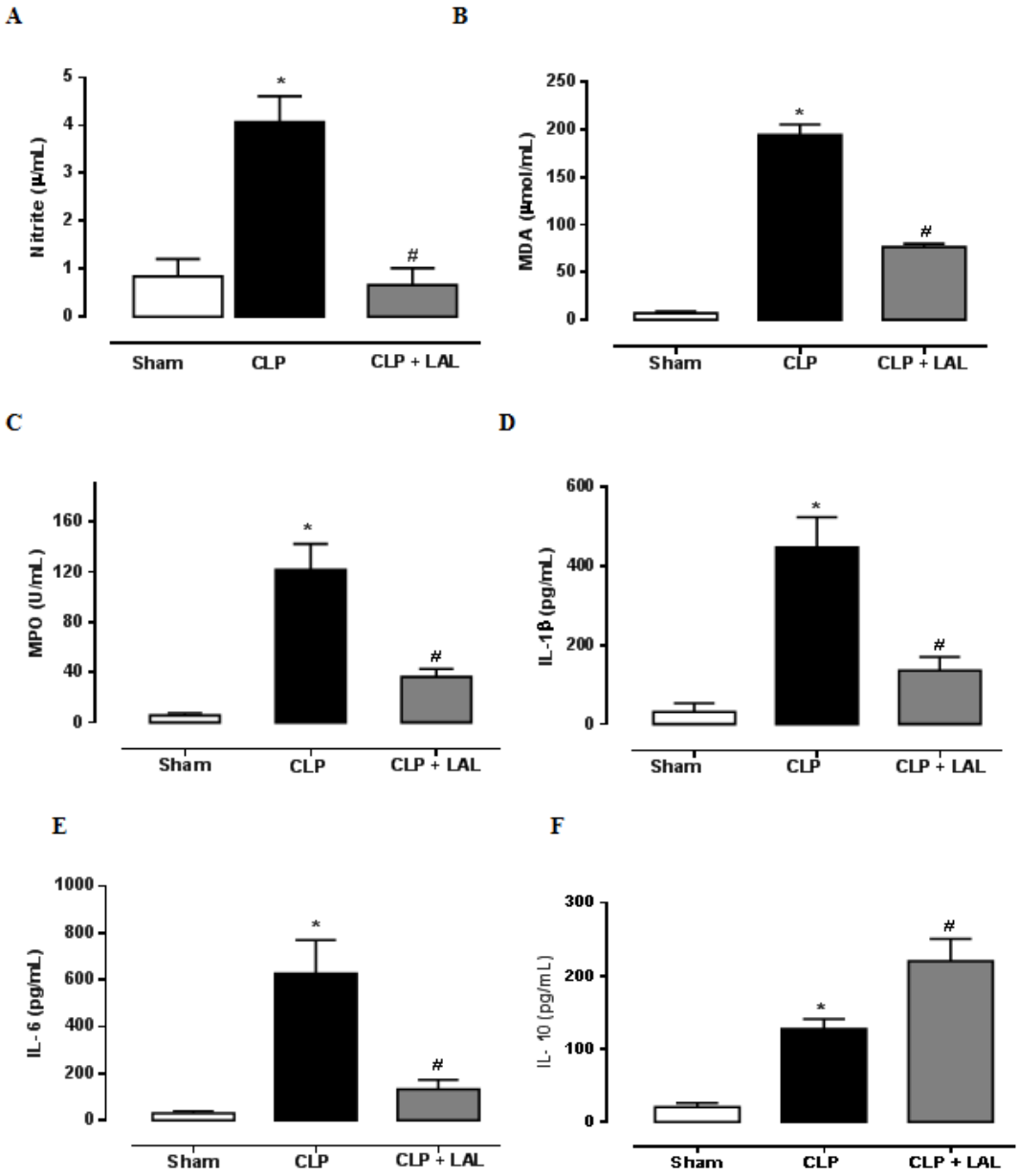

Fig. 2 LAL modulates oxidative stress and cytokine release in peritoneal fluid of septic rats. CLP or Sham animals received LAL (1 mg/kg; i.v.) 30 min before surgery. After 24 h, peritoneal fluid was collected for quantification of (A) NO_2^-, (B) MDA, (C) MPO, (D) IL-1β, (E) IL-6 and (F) IL-10. Mean ± SEM (n = 5-7).*p<0.05 *vs*. Sham; #p<0.05 *vs*. CLP

Sham: 4.3± 5.7 UMPO/mL). LAL reduced the levels of NO^{2-} by 100% (0.7 ± 1.7 µM) and MDA by 63% (76.84 ± 16 µmol/mL). LAL also reduced MPO activity by 70% (36.3 ± 5.6 UMPO/mL) (Figure 2 A, B, C). CLP increased the levels of IL-1β in 14x (447.68 ± 143.49 *vs* Sham: 31.46 ± 9.55 g/mL), IL-6 in 29x (627.35 ± 75.55 *vs* Sham: 29.6 ± 20 g/mL) and IL-10 in 6x (127.45 ± 21.45 *vs*. Sham: 20.57 ± 8.36 g/mL). LAL reduced the pro-inflammatory cytokines IL-1β (70%; 135.90 ± 35.24 g/mL) and IL-6 (78%; 133 ± 32.78 g/mL), but increased the anti-inflammatory cytokine IL-10 (43%; 220.25 ± 43.33 g/mL) (Figure 2 D, E, F).

LAL improves the relaxant and contractile response of aorta from septic rats: Sepsis reduced both aorta relaxant response to ACh (10^{-7}, $3x10^{-6}$, 10^{-5} M) by 31%, 63% and 58% (0.00035 *vs* Sham – 0.00016 AUC) and contractile response to Phe (10^{-8}-10^{-5} M) (0.00035 *vs*. Sham 0.00016 AUC) (Figure 3), that was also reflected in the decrease in potency of ACh (IC_{50} CLP 0.41 ± 0.15 *vs*. Sham 0.34 ± 0.12 µM). LAL brought the ACh-relaxant response to Sham values (0.00016 AUC) (IC_{50} 0.24 ± 0.12 µM) (Figure 3 A) and attenuated the contractile response to Phe (0.00016 AUC) of septic rats (Figure 3 B).

LAL reduces the clotting time of septic rats: Sepsis prolonged in 1.3 folds the clotting time in the PT test (35 ± 2.4 s *vs*. Sham 25.9 ± 2.1s) and LAL (21.5 ± 3.5 s) brought this time to Sham values (Figure 4A). However, LAL (22 ± 2 s) *per se* did not alter the clotting time of Sham. CLP and LAL did not alter the clotting time in the aPTT test nor the platelet aggregation (Figure 4B, C).

DISCUSSION

This study demonstrated the protective effect of *L. araripensis* lectin (LAL) in inflammatory parameters, vascular dysfunction and clotting in the rat model of sepsis induced by cecal ligation and puncture (CLP). Moderate septic animals present an effective neutrophil migration to the infectious focus and release of primary inflammatory cytokines, such as IL-1β and

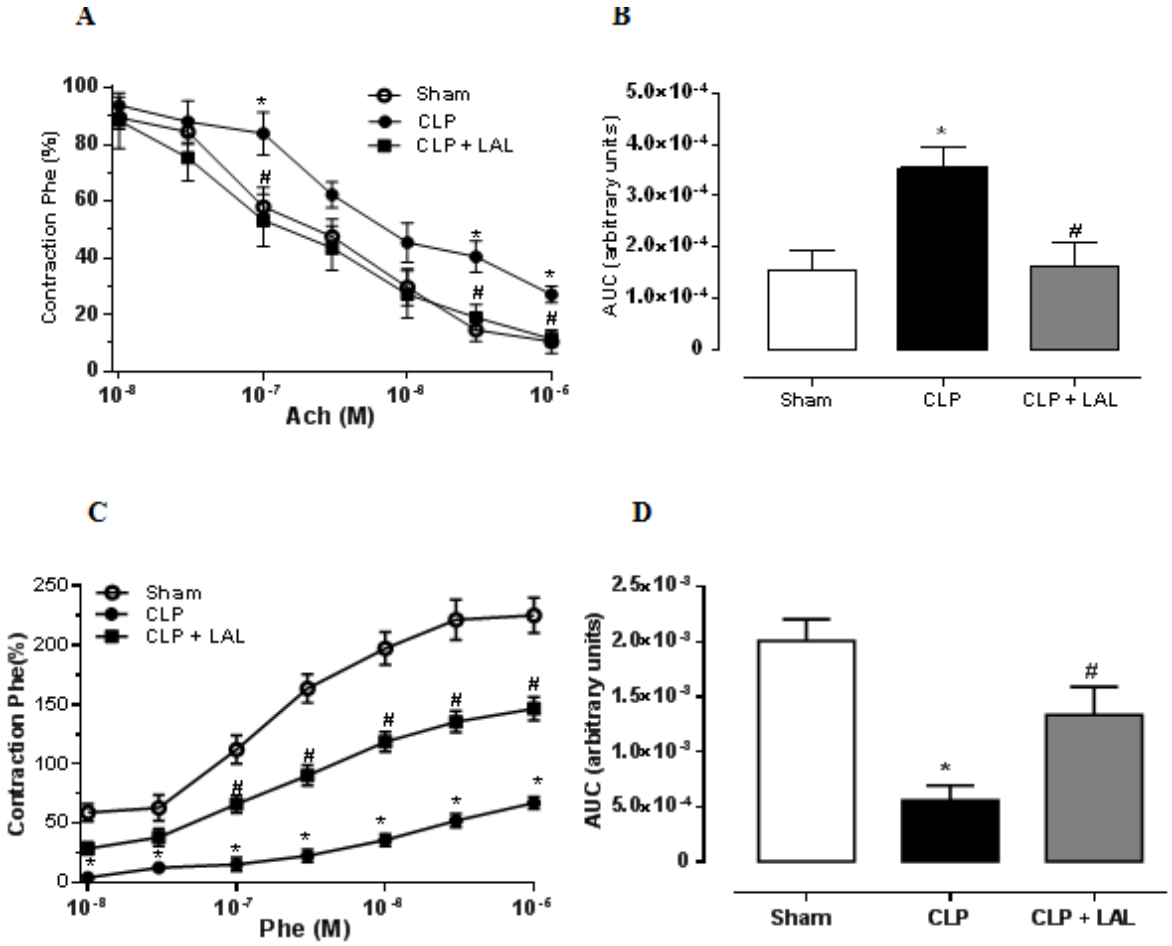

Fig. 3 LAL improves the relaxant and contractile response of aorta from septic rats. CLP or Sham animals received LAL (1 mg/kg; i.v.) 30 min before surgery. After 24 h, thoracic aorta was assessed for (A) relaxation response to ACh (10^{-8}-10^{-5} M) in Phe (0.1 µM) pre-contracted tissue; (B) AUC of ACh induced relaxation; (C) contractile response to Phe (10^{-8}-10^{-5} M); (D) AUC of Phe-induced contraction. AUC: Area under the curve; Mean ± SEM (n = 5-7).*p<0.05 *vs*. Sham; #p<0.05 *vs*. CLP

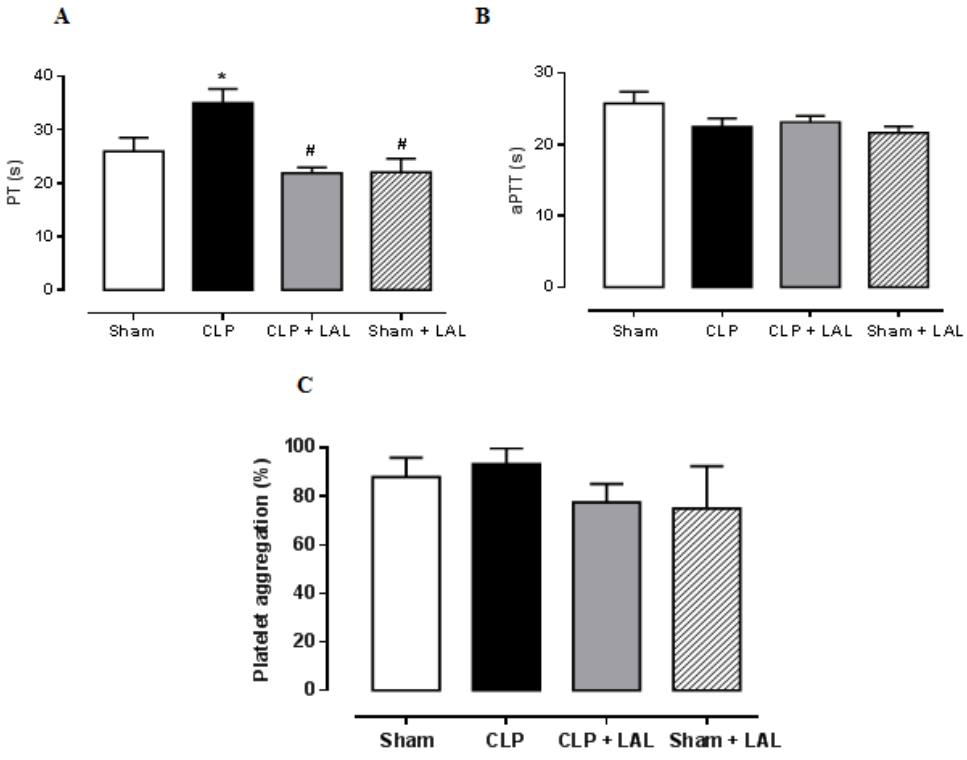

Fig. 4 LAL reduces the clotting time of septic rats.CLP or Sham animals received LAL (1 mg/kg; i.v.) 30 min before surgery. After 24 h, blood was collected for assessment of clotting. (A) PT; (B) aPTT; (C) platelet aggregation. Mean ± SEM (n = 5-7).*p<0.05 *vs*. Sham; #p<0.05 *vs*. CLP

TNF-α, associated to the increased microbicidal activity and sepsis prognosis (Gyawali, Ramakrishna, Dhamoon, 2019; Amersfoort, Berkel, Kuiper, 2003). These cytokines promote deregulated leukocyte recruitment and synthesis of other cytokines, lipid mediators (prostaglandins, platelet aggregation factor), oxidative stress and expression of adhesion molecules (Amersfoort, Berkel, Kuiper, 2003; Hong *et al*., 2013). In our study, 24 h after sepsis induction, neutrophil migration and MPO activity were shown to be increased in the rat peritoneal fluid, along with the concentration of IL-6, IL-1β and IL-10. LAL reduced neutrophil migration, MPO activity and inflammatory cytokines (IL-6, IL-1β), besides increased the anti-inflammatory IL-10. These results are in line with the literature of sepsis, both in humans and in experimental models, describing high concentration of IL-6, which is correlated to the disease severity, multiple organ dysfunctions and lethal outcome (Remick*et al*., 2005). Besides, the use of the antibody anti-IL-10 has been shown to be crucial for sepsis outcome in CLP model. On the other hand, IL-10 exhibits both protective and deleterious effect during sepsis (Song *et al*., 1999) and the high IL-6/IL-10 ratio has been associated to worse prognosis in septic patients (Taniguchi *et al*., 1999). LAL also reduced the oxidative stress generated by high levels of MDA and nitrite and increased MPO activity in septic animals, which could protect DNA and endothelial cell damage, cytokine formation and neutrophil recruitment. In this line, the literature has shown that septic rats treated with antioxidants present reduced MPO activity and lipid peroxidation in liver, kidney, heart, lung and diaphragm (Ritter *et al*., 2003). The oxidative stress has been also associated to the severity and mortality of septic patients (Ritter *et al*., 2003; Lorente *et al*., 2013).

Cardiovascular alterations in septic shock are associated to excessive NO production in animals with reduces vascular tonus caused by endotoxins or inflammatory cytokines (Cruz, Kenyon, Sandrock, 2009). In our study, the septic animals showed reduced contractile and relaxant responses to Phe and ACh, respectively, characterizing the endothelial dysfunction in the preliminary stage of sepsis (Matsuda, Hattori, 2007; Asano *et al*., 2015). LAL recovered aorta responsiveness to Phe and ACh, suggesting a controlled production of NO via eNOS activation. These data are in agreement with previous studies that demonstrated a decreased concentration of peritoneal nitrite and the LAL vasorelaxant effect via NO in endothelized aorta of non-septic rats (Pires *et al*., 2017). Our results demonstrated that sepsis increases the coagulation time in the PT test. Accordingly, recent studies have shown that the extrinsic pathway is accountable for the onset of coagulation in sepsis, characterizing a factor tissue (FT) dependent process and that PT prolongation is often encountered in septic patients (Collins *et al*., 2006). Besides, the literature had shown that sepsis can be correlated to low levels of factor VII, factor II, factor V, antithrombin and protein C, but to high values of fibrinogen and factor VIII, leading to PT and aPTT prolongation, but normal platelet counts and normal thromboelastometry (Collins *et al*., 2006; Lukas *et al*., 2018). The coagulation system is commonly activated in sepsis as a result of cross-reactions with inflammatory system, it means that coagulation in sepsis is not constant and may be changed dynamically along the time. Studies have suggested that IL-6, which has been found to be increased in the present study, is accounted for thrombin generation that also initiates fibrinolysis (Remick *et al*., 2005; Levi, Van der Poll, 2015). Thus, one factor that can explain the re-established coagulation time, in the extrinsic pathway, by LAL is the reduction of IL-6 levels. The re-established coagulation time could also be explained by the re-structuration of vascular endothelium, the reduction of levels of nitrite and oxidative stress, thus decreasing TF. It is important to highlight the innovative aspect of this study, since there are no reports evaluating leguminous lectins in sepsis. In addition, previous studies showing high tolerance of rats and mice after LAL treatment during seven days (Pires *et al*., 2016; Pires *et al*., 2019b), validate our present data in which LAL did not alter the physiological conditions of sham animals.

Conclusion

LAL protects inflammation, bleeding (tissue factor/common pathway of coagulation) and vascular dysfunction in rat polymicrobial sepsis.

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