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Pulmonary and muscle profile in pneumosepsis: A temporal analysis of inflammatory markers

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ABSTRACT

In sepsis, greater understanding of the inflammatory mechanism involved would provide insights into the condition and into its extension to the muscular apparatus in critically ill patients. Therefore, this study evaluates the inflammatory profile of pneumosepsis induced by *Klebsiella pneumoniae* (*K.p.*) in lungs and skeletal muscles during the first 72 h. Male BALB/c mice were divided into 4 groups, submitted to intratracheal inoculation of *K.p.* at a concentration of 2×10^8 (PS) or PBS, and assessed after 24 (PS24), 48 (PS48) and 72 (PS72) hours. The Maximum Physical Capacity Test (MPCT) was performed before and after induction. Pulmonary inflammation was assessed by total cell number, nitric oxide levels (NOx), IL-1β and TNF-α levels in bronchoalveolar lavage fluid (BALF); inflammation and muscle trophism were evaluated by the levels of TNF-α, IL-6, TGF-β and BDNF by ELISA and NF-κB by western blotting in muscle tissue. Cells and colony forming units (CFU) were also analyzed in blood samples. The PS groups showed an increase in total cells in the BALF ($p < 0.05$), as well in the number of granulocytes in the blood ($p < 0.05$) and a decrease in performance in the MPCT ($p < 0.05$). NOx levels showed significant increase in PS72, when compared to Control group ($p = 0.03$). The PS24 showed a significant increase lung in TNF-α levels ($p < 0.001$) and in CFU ($p = 0.013$). We observed an increase in muscular IL-6 and nuclear NF-κB levels in PS24 group, when compared to PS48 and Control groups ($p < 0.05$). Nevertheless, mild signs of injury in the skeletal muscle tissue does not support the idea of an early muscular injury in this experimental model, suggesting that the low performance of the animals during the MPCT may be related to lung inflammation.

1. Introduction

Sepsis is an important public health problem, particularity in the intensive care units (ICU), associated to high incidence, morbidity and mortality rates [1,2]. Sepsis has been the focus of several clinical and experimental studies to address diverse mechanisms ensuing intense systemic inflammatory processes, as well as its short and long-term repercussions and disabling effects. Septic individuals present worse clinical outcomes, particularly in the physical and cognitive domains, as compared to other types of hospitalized patients [3–5].

Sepsis results in a complex interaction between the infecting microorganism and the host's proinflammatory and procoagulant immune response, and, thus, may manifest itself in a severity spectrum, such as septic shock and multiple organ failure [6]. The lungs are the most common site for the development of sepsis, accounting for about 50% of all sources of infection [7,8]. The respiratory tract is particularly susceptible to infection, being one of the first sites to be exposed by pathogens particularly due to its extensive internal surface [9].

The skeletal muscle system is also intensely affected in sepsis of pulmonary origin or with Acute Respiratory Distress Syndrome (ARDS), either in clinical or experimental settings. Animal models of sepsis demonstrate significant muscle atrophy, which can concomitantly lead

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to acute lung injury [10,11]. Inflammation of this nature is characterized primarily by the influx of neutrophils and macrophages into the lung leading to a systemic proinflammatory cytokines burden. Among other, IL-1β, TNF-α and IL-6 are known to increase muscle proteins degradation [10]. These proinflammatory cytokines can also activate pathways controlling skeletal muscle mass, like NF-κB, involved in catabolic pathways, enhancing levels of brain-derived neurotrophic factor (BDNF), which expression in muscle is increased in response to injury [12,13]. In addition, the activation of TGFβ pathways indirectly limits the skeletal muscle hypertrophy signaling [14]. In fact, patients surviving sepsis present long-term decrease in the quality of life, which may be related a dysfunction known as polyneuromyopathy of the critical patient, along with functional and cognitive impairment [1,2]. Recovery from these complications can take months to years. Some studies have shown that a range of risk factors may be associated with the development of post-sepsis morbidities, especially muscle weakness [15]. De Jonghe et al. [16], for example, reported that among several independent predictors for the development of muscle weakness, sepsis was considered a predominant risk factor. These complications may also result in decreased weaning from mechanical ventilation, increasing the patient's ICU time, which can further increase functional disabilities [17].

Considering that animal models of sepsis demonstrate significant muscle atrophy, and that muscle protein degradation is thought to be a process mediated proinflammatory cytokines, this study aims to evaluate the pulmonary and muscular inflammatory profiles in sepsis induced by *Klebsiella pneumoniae* (*K.p.*), with particular interest in phenomena occurring along the first 72 experimental hours.

2. Material and methods

2.1. Approval from the Ethics Committee

All the animals received care in accordance with EU Directive 2010/63/EU [18], which provides guidelines for animal experiments and ARRIVE (Animal Research: Reporting In Vivo Experiments) [19] and is approved by the Ethics Committee on Animal Use of the Federal University of Santa Catarina (No. PP00745).

The animals were obtained from the central laboratory of the Federal University of Santa Catarina/UFSC and maintained under controlled conditions of temperature (21–23 °C), and luminosity (12 h light cycle/12 h dark) in the animal maintenance facility of the Laboratory of Pain and Inflammation of the Federal University of Santa Catarina (LANDI/UFSC). Food and water were provided "*ad libitum*".

2.2. Experimental groups

Adult male BALB/c mice, aged 6–8 weeks, weighing approximately 20–28 g were used. The inflammatory characteristics of the pneumosepsis induced by *Klebsiella pneumoniae* (*K.p.*) were analyzed in the lungs and peripheral muscles at three periods, namely: 24, 48 and 72 h after induction. For that purpose, the animals were divided into the following experimental groups: Control (C) (n = 10), pneumosepsis 24 h (PS 24) (n = 10), pneumosepsis 48 h (PS 48) (n = 10) and pneumosepsis 72H (PS 72) (n = 10) (Fig. 1).

2.3. Maximum physical capacity test (MPCT)

The animals were submitted to a maximum stress test - Maximum Physical Ability Test - (MPCT) on a treadmill adapted for mice (executive model 550 EX-1, EMBREEX®, Brusque, SC, Brazil), prior to and after pneumosepsis induction by *K.p.*, according to the protocol described by Vieira et al. and Olivo et al. [20,21].

The MPCT consisted of a 5 min warm-up at 0.2 km/h, after which the velocity was increased 0.1 km/h every 2.5 min until exhaustion (observed when the mice could not continue running after slight mechanical stimuli). Prior to the test, the animals underwent three days of adaptation on the treadmill, performing 10 min of activity at a speed of 0.2 km/h, according to the protocol described in Fig. 1.

2.4. Experimental model of pneumosepsis

The bacterium used in this experimental protocol was *Klebsiella pneumoniae* (ATCC 700603 - American Type Culture Collection, Rockville, MD). The aliquots were kindly provided by the Nitric Oxide Laboratory of the Pharmacology sector of the Federal University of Santa Catarina.

2.4.1. Mortality curve

In order to evaluate the pneumosepsis profile, a separate experimental set was conducted to analyze the mortality curve. For this, 5 animals from each group were used, inoculated with 2×10^8 or 1×10^9 *K.p.* Colony Forming Units (CFU) per animal. At the end of 144 h of observation, the number of deaths in each group was evaluated and the survival percentage was plotted (Fig. 2).

2.4.2. Quantification of the bacteria

As *per* Sordi et al. [22,23], the contents of a microtube of lyophilized bacteria were suspended in 45 mL of Brain Heart Infusion (BHI) within a falcon tube and incubated for 18 h at 37 °C. The bacteria were washed and, at the end, the resulting pellet was suspended in 0.5 mL of sterile phosphate buffered saline (PBS) and placed in microtubes. Serial dilutions were then performed and optical densities (OD) determined at 600 nm. Concurrently, 10 μL of the dilutions were plated in duplicate on Mueller-Hinton agar under sterile conditions and incubated at 37 °C for 24 h. After which time, the CFUs were determined and the amount per milliliter of each dilution was calculated. Linear regression calculation was performed, correlating the OD and CFU/mL used, to estimate the number of CFUs from all the aliquots.

2.4.3. K.p. inoculation of the animals

Individual aliquots were thawed and washed 2 times in sterile PBS and placed in BHI broth and incubated for 18 h at 37 °C. The broth was centrifuged and the resulting pellet washed twice and suspended in 0.5 mL of sterile PBS. The bacterial concentration was determined by interpolating the measured absorbance at 600 nm in the regression. The bacterial suspension was diluted in sterile PBS at the concentration desired for inoculation.

The animals were anesthetized with a solution composed of ketamine (3 mL), xylazine (1.8 mL) and PBS (25.2 mL) and placed in an inclined (45°) supine position. Aseptically, a 5 mm incision was made in the skin of the ventral region of the neck and the musculature was gently parted, with the identification of the trachea for the injection of 0.05 mL of the intratracheal bacterial suspension. 2×10^8 CFU of *K.p.* per animal were inoculated. After the procedure, the skin was sutured and the animals received subcutaneous PBS (30 mL/kg) for fluid replacement, and remained in a warm environment until recovery from the surgery. The animals from the Control group underwent the same surgical procedures, however, receiving intra-tracheal injection of sterile PBS (0.05 mL) [22,23].

2.5. Blood leukocyte count

Within the stipulated period for each group (24, 48 or 72 h), the animals were anesthetized as described above. Through an opening in the thoracic cavity, approximately 1 mL of blood was collected via hepatic puncture in a heparinized syringe (5 IU/ml blood). The mice were then sacrificed by rapid sectioning of the abdominal aorta. The blood sample was subjected to cell counting using an automated hematology counter (Horiba Abx Micros 60, Shandong, China).

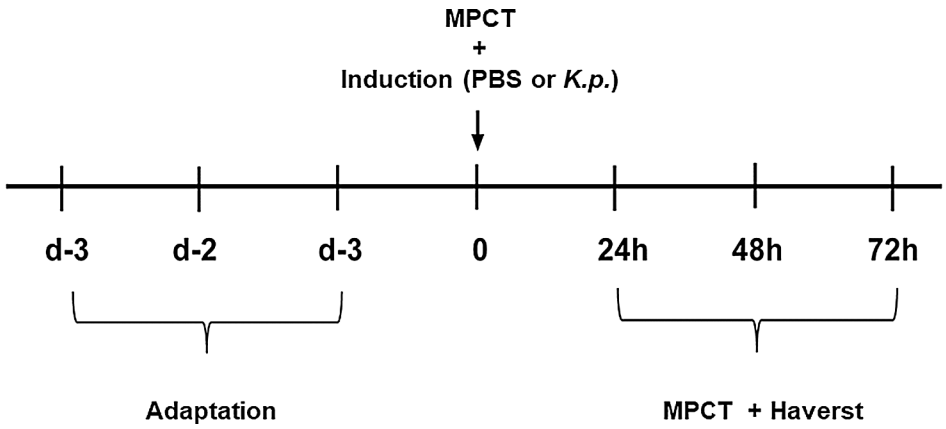

Fig. 1. Timeline of the experimental protocol. The animals underwent treadmill adaptation for 3 days. At d0, tracheal inoculation of saline or *K.p.* at a concentration of 2×10^8/animal was performed. Euthanasia occurred at 24, 48 and 72 h after *K.p.* sepsis induction. In the control group, *K.p.* was replaced by PBS.

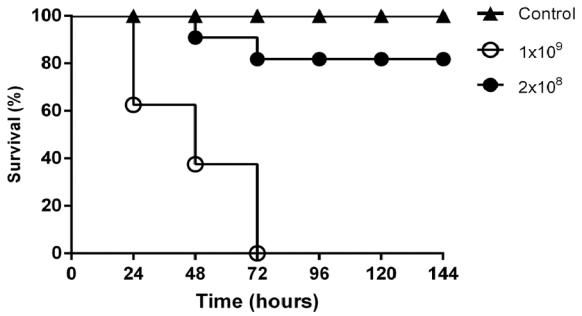

Fig. 2. The percentage of survival of the mice was recorded in a period of 144 h, obtained through a second experimental set.

2.6. Quantification of bacterial colony-forming units (CFU) in the blood samples

From obtained blood sample, 10 µL were separated and seeded in Petri dishes containing Mueller-Hinton agar medium. After seeding, the plates were incubated at 37 °C for 24 h and the number of colonies, expressed as a logarithm of colony forming units/mL (log of CFU/mL) was counted [22].

2.7. Collection and analysis of the bronchoalveolar lavage (BAL)

After blood collection, a tracheostomy was performed to collect the BAL. The lungs were gently washed three times with 0.5 mL of PBS using a tracheal cannula. Once collected, the BAL was centrifuged at 1000 rpm, at a temperature of 4 °C, for 10 min. The supernatant was then stored at −80 °C and the cell button was re-suspended in 500 µL of PBS in order to count the total number of cells using a hematocytometer (Neubauer Chamber) [20,21].

After the BAL collection procedure, the chest was opened, and the lungs and peripheral musculature (gastrocnemius and soleus) were removed and stored at −80 °C for later analysis.

2.8. Indirect analysis of the nitric oxide in the BAL

Nitric oxide (NO) levels were quantified by measuring the sum of nitrate and nitrite (NOx) concentrations using Griess reagent (1% sulfanilamide in 10% phosphoric acid/0.1% of alpha-naphthyl-ethylenediamine in Milli-Q water) as previously described [24]. The BAL was deproteinized by the addition of zinc sulfate (2%), while to convert the nitrate to nitrite, the samples were incubated at 37 °C for 2 h in the presence of nitrate reductase expressed in *Escherichia coli* cultured in anaerobiosis. Following the enzymatic conversion of nitrate to nitrite, the samples were centrifuged to remove the bacteria, and 100 µL of the supernatant was mixed with the same volume of Griess reagent, producing lilac color. The assay was performed in 96-well plates, and the reading carried out at 540 nm in a microplate reader (Infinite M200, Tecan Group Ltd, Switzerland). Standard nitrite and nitrate curves (0–150 µM) were performed simultaneously. As the conversion rate of nitrate to nitrite was always greater than 90%, no corrections were made to the results, which were expressed as µM of NOx [23].

2.9. Measuring the cytokine levels

The dissected soleus and gastrocnemius muscle tissue samples were homogenized with an Ultra-Turrax homogenizer (IKA T18 basic, IKA®, Germany) in a PBS solution containing: Tween 20 (0.05%), phenylmethylsulfonyl fluoride (PMSF), 1 mM, ethylenediaminetetraacetic acid, 10 mM acetic acid (EDTA), 2 ng/mL aprotinin and 0.1 mM benzamethonium chloride (Sigma Chemical Company, St. Louis, MO, USA). The homogenates were transferred to 1.5 mL eppendorf tubes, centrifuged at 3000*g* for 10 min at 4 °C, and the supernatant obtained was stored at −70 °C until later analysis. Total protein was measured in the supernatant using the Bradford method, and the levels of tumor necrosis factor (TNF-α), brain-derived neurotrophic factor (BDNF), interleukin-6 (IL-6) and transforming growth factor beta (TGF-β) from the peripheral muscles were analyzed.

100 µL BAL samples were also used to analyze the cytokines and measure the TNF-α and IL-1β levels. All analyses were performed using mouse ELISA kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

Cytokine levels were obtained by interpolation from a standard curve by colorimetric measurement at 450 nm (540 nm wavelength correction) in the ELISA plate reader (Berthold Technologies - Apollo 8 - LB 912, Bad Wildbad, KG, Germany). The results from the BAL and muscle samples were expressed as pg/ml and pg/mg protein, respectively.

2.10. Western blot for NF-κB

Gastrocnemium tissues were quickly frozen in liquid nitrogen, homogenized in ice-cold lysis buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 5% glycerol, 1% Triton X-100, 0.2 mM Na_3VO_4, 40 mM beta-glycerophosphate containing 10 µg/ml each of aprotinin, leupeptin, soy bean trypsin inhibitor, and 1 mM PMSF), centrifuged at 10,000g and the supernatant collected. The BCA protein assay kit was used to determine protein concentrations. Protein samples (20 µg/well) were subjected to denaturing gel electrophoresis (SDS/PAGE, 10% gel) and run in the Mini-PROTEAN® Tetra cell apparatus (Bio-Rad, CA, USA). After electrophoresis, proteins were electro-transferred to nitrocellulose membranes (Hybond;

Amersham Biosciences, NJ). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk (prepared in TBS-T buffer, pH 7.4; concentration in mmol/L: 20 Tris–HCl, 137 NaCl; and 0.1% Tween 20), and subjected to incubation (overnight, at 4 °C) with primary antibodies against NF-κB (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following washing, membranes were incubated with rabbit peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were exposed to HPR substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and immune complexes were visualized by chemiluminescence using Chemidoc MP System (Bio-Rad Laboratories). Bands were quantified by densitometry using the software from the manufacturer.

2.11. Statistical analysis

Data were analyzed using Prisma Graphpad 6.0 software (California, USA, 2005). The Kolmogorov-Smirnov test was used to assess the distribution of normality and the data were submitted to one-way ANOVA, followed by the Dunnett's post-test, for comparison of all groups with the group control. Additionally, in the analysis of the total number of cells in the BAL, the Holm-Sidak post-test was performed to analyze multiple comparisons between the groups.

In the MPCT analysis, the *t*-test was used for intra-group comparison in the periods before and after inoculation with the bacteria. Significance levels were adjusted to 5% ($p < 0.05$). Values were expressed as mean ± SD.

3. Results

3.1. Analysis of survival after K.p. inoculation

The severity of lung injury caused by *K.p.* inoculation in the lungs was analyzed by comparing two concentrations, 2×10^8 and 1×10^9. In this experimental protocol, all animals inoculated with 1×10^9 died within 72 h, while 80% of animals inoculated with 2×10^8 survived up to 144 h of follow-up (Fig. 2).

3.2. K.p. inoculation at 2×10^8 resulted in an increase in the total cell count and NOx in the BAL

Pulmonary inflammation was also evaluated through cell quantification and NOx levels in the BAL (Fig. 3A and 3B). Animals from the pneumosepsis groups presented a statistically significant increase in the total number of cells ($p < 0.05$) after 24, 48, and 72 h, when compared to Control group animals. The total number of cells was higher at 48 and 72 h, as compared to 24 h. Regarding NOx levels in the BAL, a statistically significant increase was only observed when the PS72 group was compared to the Control group ($p < 0.05$).

3.3. K.p. inoculation at 2×10^8 resulted in an increase in the pro-inflammatory cytokine levels in the lungs

The levels of proinflammatory cytokines in the BAL were evaluated (Fig. 4A and B), and demonstrated a statistically significant increase in the levels of TNF-α in the PS24 group, when compared to the Control group ($p < 0.001$; Fig. 4A). In addition, the PS72 group also presented a statistically significant increase in IL-1β levels when compared to the Control group ($p < 0.001$; Fig. 4B).

3.4. K.p. Inoculation at 2×10^8 resulted in an increase in the number of granulocytes and CFU

Blood cellularity was evaluated through the total number of granulocytes, which presented a statistically significant increase in all the Pneumosepsis groups (Fig. 5A) when compared to the Control group. The number of CFUs also showed an increase in the first 24 h, when compared to the Control group ($p < 0.05$) (Fig. 5B), which was not the case at 48 and 72 h after Pneumosepsis induction.

3.5. K.p. inoculation at 2×10^8 did not result in persistent inflammation status in muscle tissue

Muscle inflammation were assessed through tissue levels of TNF-α, IL-6, TGF-β, BDNF and NF-Kb (Fig. 6). IL-6 (Fig. 6B) and nuclear NF-KB (Fig. 6E) showed an increase in PS24 group when compared to PS48 and Control group ($p = 0.02$). We did not observe any statistic difference ($p > 0.05$) in TNF-α (Fig. 6A), TGF-β (Fig. 6C) and BDNF (Fig. 6D).

3.6. The physical performance of the animals diminished after K.p. inoculation

The functional capacity of the animals was assessed using the MPCT (Fig. 7A and B). The animals from the Pneumosepsis groups showed a statistically significant drop in distance traveled and velocity after intratracheal inoculation of *K.p.*, when compared to the initial evaluation (PS 24, $p < 0.001$; PS48, $p = 0.021$ and PS72, $p = 0.002$, in the evaluation of distance traveled and PS24, $p < 0.001$; PS48, $p = 0.022$ and PS72, $p = 0.004$, respectively).

4. Discussion

One of the great challenges related to sepsis has been the development of an understanding of the various mechanisms involved in the intense inflammatory process, its systemic repercussions, as well as the short and long-term debilitating effects. Despite their importance, few studies have investigated the muscular and pulmonary profiles in sepsis of respiratory origin over time.

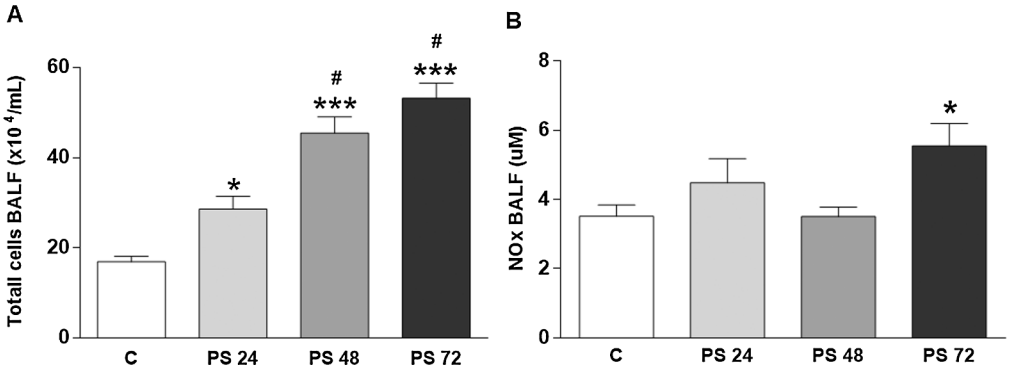

Fig. 3. Effects of pneumosepsis-induction by *K.p.* at 2×10^8 on the total number of cells (A) and the nitric oxide levels (B) in the BAL. (A). Statistical difference was $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared to the control group, and $\#p < 0.05$ when compared to the PS24.

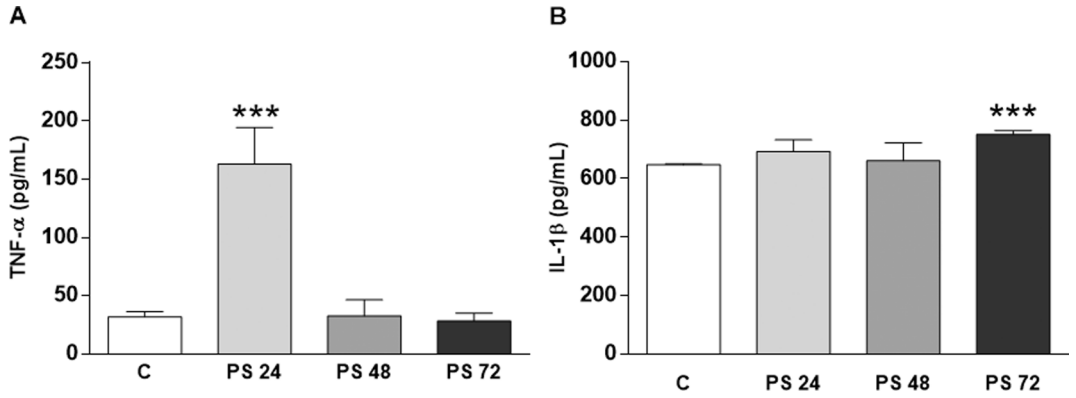

Fig. 4. Effects of pneumosepsis induction by *K.p.* at 2×10^8 on the levels of TNF-α (A) and IL-1β (B) in the BAL. (A). Statistical difference was $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared to the control group.

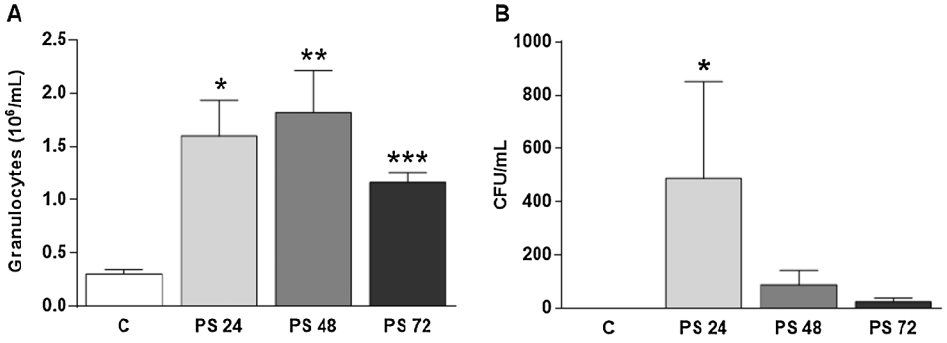

Fig. 5. Effects of pneumosepsis induction by *K.p.* at 2×10^8 on the number of granulocytes (A) and CFU (B) in the blood (A). Statistical difference was $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared to the control group.

In this experimental model of *K.p.*-induced pneumosepsis, within a 72-h analysis interval, all the animals inoculated with the bacterium presented a decreased performance in the MPCT, either in terms of speed or distance traveled. In spite of this, the biochemical analyzes in the gastrocnemium muscle showed some significant differences between the studied groups. On the other hand, animals with pneumosepsis presented a characteristic inflammatory process in the BAL, with increased total cellularity, nitric oxide, as measured by NOx levels, and

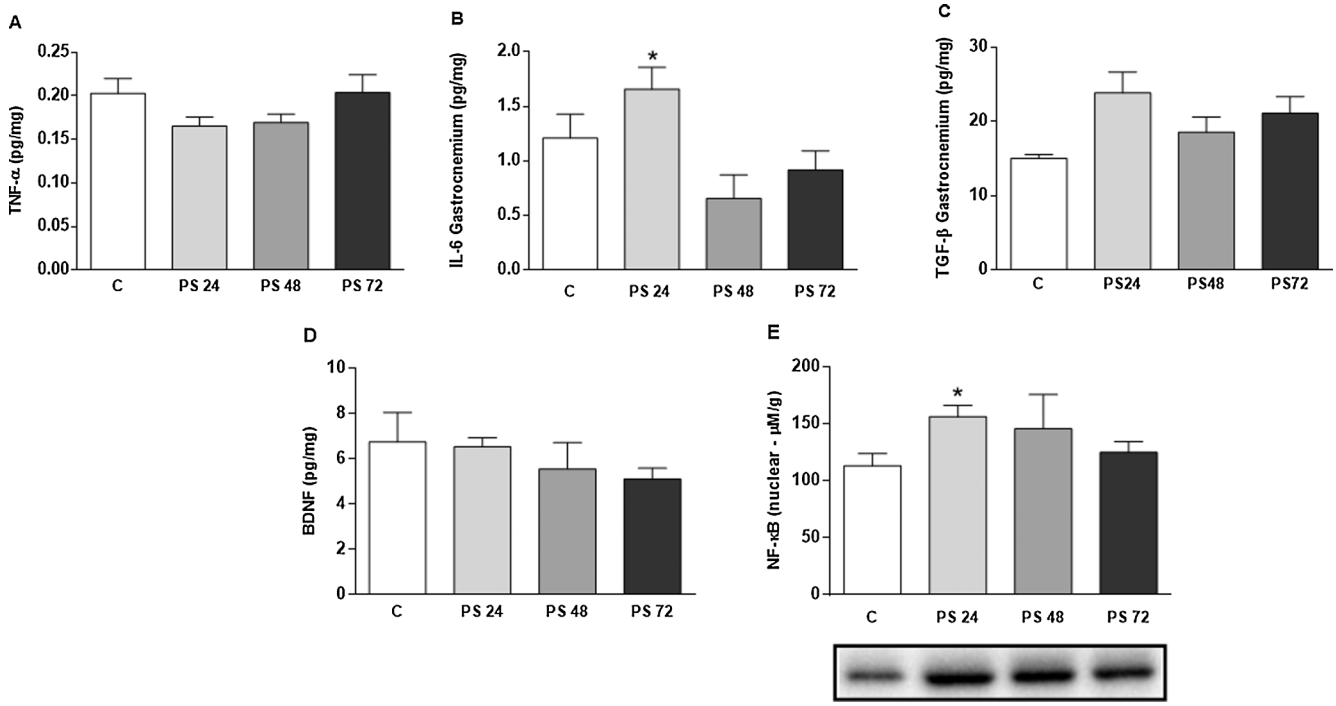

Fig. 6. Effects of pneumosepsis induction by *K.p.* at 2×10^8 on TNF-α (A), IL-6 (B), TGF-β (C), BDNF (D) and NF-Kb nuclear (E) in gastrocnemium tissue. Statistical difference was $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared to the control group.

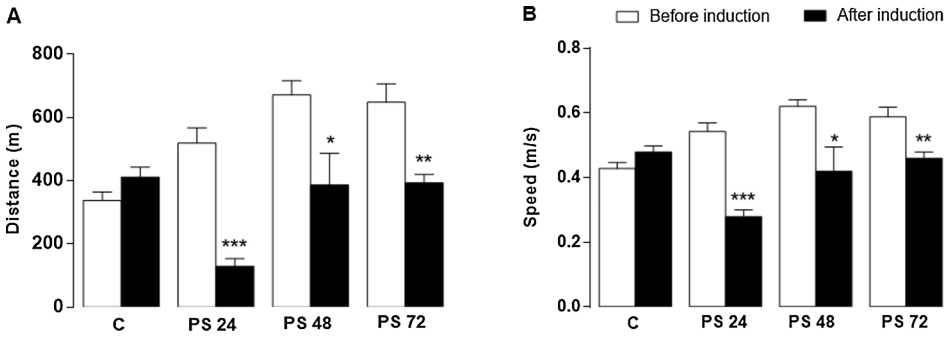

Fig. 7. Effects of pneumosepsis induction by *K.p.* at 2×10^8 on the distance travelled and (A) and Velocity (B) in the MPCT. Statistical difference was $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared to the control group.

the proinflammatory cytokines TNF-α and IL-1β. In addition, a systemic increase in the number of granulocytes and *K.p.* CFU of inoculated animals was also observed, characterizing a bacteremia in the first 24 h following inoculation.

This cellular displacement to BAL, as observed in septic animals, can be partly explained by the activity of nitric oxide (NO). According to Förstermann and Sessa [26], significant amounts of NO secreted by macrophages and activated neutrophils can lead to damage to the surrounding tissue, and increase the influx of inflammatory cells in the lung [25]. In addition to having microbicidal action, nitric oxide is involved in practically all host's responses to infection, such as increased vascular permeability, the migration and activation of leukocytes, and vasodilation, whether local or systemic [27–32].

In contrast to Sordi et al. [23], who, in a similar experimental model, observed an increase in the NOx level in the first 24 h in the lung lavage, in this study, NOx levels in the BAL showed a significant increase only 72 h after *K.p.* induction, just at the same moment of greatest cellular influx into the lungs. Bacterial concentrations differed, Sordi et al. [23] used a concentration of 4×10^8, while in this study we opted to use 2×10^8 cells. It is suggested that these temporal changes in BAL, specially the BAL NOx peak, may be dependent the intensity of *K.p.* stimulus.

NO production can also be mediated by TNF-α, a proinflammatory cytokine produced primarily by activated macrophages [29]. TNF-α is the main mediator of the acute inflammatory response to gram-negative bacteria, such as *Klebsiella pneumoniae*, and is responsible for many serious infections [33]. Some studies point to TNF-α as an indicator of inflammation or poor prognosis in septic conditions [32,34,35]. The main physiological function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to infection sites, and to activate those cells to eradicate microorganisms [30]. Thus, the release of this cytokine at the site of the infection occurs within the first few hours of sepsis, as demonstrated by Sordi et al. [23], which was confirmed by our results.

In spite of the high cellular concentration in the lungs at later time (72 h), this study also found a significant increase of blood granulocytes in the interval between 24 and 72 h after *K.p* instillation, as well as a rapid increase in the number of systemic CFU (24 h). Therefore, these data indicate that in this experimental protocol, there was a systemic response to local injury. Although the presence of bacteria in the bloodstream was found to be transient, it is well described that bacteremia is not essential for the development of sepsis, precisely because of the rapid clearance of the bacterium from the bloodstream, in addition to its preference for certain organs such as the lungs, for example [23,36,37].

In a joint action with TNF-α, IL-1β is known to act on the natural immunity and on the inflammation. Its production may be induced by bacterial products, such as LPS, as well as by other cytokines, such as TNF-α itself [33]. The cytokine IL-1β is synthesized by macrophages, and has also been identified as being capable of inhibiting protein synthesis and regulating iNOS expression, in addition to stimulating prostaglandin production [11]. Due to its interrelationship with a range of inflammatory mediators, it is also known that IL-1β, at high plasma concentrations, may play an important role as a biomarker, presenting good correlation with the clinical outcomes of septic patients [11]. In our experimental model, it was observed that, while TNF-α levels in the infectious focus were elevated in the first 24 h, suggesting an important role in the genesis of the inflammatory process. IL-1β levels appear to increase gradually, especially after 72 h following bacterial instillation, as described by Friedrich [11].

In addition to its inflammatory role in sepsis, some evidence suggests that TNF-α may be expressed in muscle fibers and promote muscle atrophy and weakness in critically ill patients. TNF-α has been extensively studied in sepsis because it causes pronounced damaging effects in muscles, such as the induction of muscle proteolysis, atrophy and activation of proteolytic systems such as ubiquitin-proteasome (UPS) [38].

Studies in experimental models of pulmonary sepsis and some models of Acute Respiratory Distress Syndrome (ARDS) have shown the development of important musculoskeletal atrophy in animals [11]. The onset of muscle atrophy in these models seems to occur temporally and simultaneously to acute lung injury (characterized by the influx of neutrophils and macrophages into the lung, in addition to the increased production, release and activation of proinflammatory cytokines), and systemic inflammation [10,11].

Our analyses of the muscle levels of TNF-α, TGF-β, BDNF and cytoplasmatic NF-ƙB found no significant difference between 24 and 72 h after pneumosepsis. It is possible that an initial 'latency' phenomenon can precede muscle injury, as described by Friedrich and collaborators [11]. Nevertheless, our results showed a rapid (24 h) increase in IL-6 levels in muscle tissue, as well as in the nuclear NF-kB, suggesting that in our sepsis protocol, there is distinctive pathways involved in muscle injury, decreasing trophic factors and increasing catabolic mechanisms, as described by Egerman and Glass [14]. By contrast, Supinsku and Leigh [39] reported that the administration of endotoxin rapidly induces large reductions in the ability to generate contractile proteins in slow and fast fibers of both the limbs and respiratory muscles. Additionally, Mofarrahi et al. [40] reported that the induction of severe sepsis can trigger morphological and functional lesions in mitochondria, associated with a significant induction of autophagy in skeletal muscles. According to those authors, however, responses of this magnitude may be dependent on the pattern of activation and the type of composition of the fiber in various skeletal muscles.

The results showing decreased distance traveled and velocity during the MPCT, observed in animals inoculated with *K.p.*, is *a priori* due to the observed pulmonary inflammation, and to a lesser degree to any degree of muscular degradation. Alike, Files et al. [10] observed that ALI itself seemed to cause type II myofiber atrophy in soleus, with type I myofibers being preserved. In addition, the authors observed an atrophy in both type I and type II myofibers of the diaphragm, which could be an additional factor to contribute to decreased physical performance in septic mice.

Therefore, in this experimental model, it was observed that, during a 72-hour analysis window, there was a septic inflammatory process in the lungs, which possibly resulted in a decrease in the performance of the animals during the MPCT.

Competing interests

There is no conflict of interest.

Author contributions

DCHK, AD, KCB and JJP: conceived the study design; data collection, analysis and interpretation of data, and wrote the manuscript. GHC, AHSS, MB, AMV: collected data. VVH: Prepared the bacteria and analyzed data. FB: Analyzed data. DFM and ARSS: Provided technical support and supervised the project. All authors have approved the final version of the article and agree to provide all clarifications to ensure the completeness and accuracy of the research.

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