Full Length Research Paper

Prophylactic administration of a Propionibacterium acnes-killed preparation increases survival in animals with polymicrobial sepsis via the nitric oxide and TNF-α pathway

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Propionibacterium acnes (P. acnes) is a gram-positive anaerobic microorganism present in human skin, and has being widely used in clinical trials as a suitable candidate for therapeutic approach to sepsis. A previous study performed by our research group demonstrated that a P. acnes killed preparation had an important immunomodulatory role in severe sepsis. Hence, this study we evaluated the immunomodulatory effect of P. acnes preparation on sub-lethal sepsis using a clinically relevant animal model of polymicrobial sepsis. Cecal ligation and puncture (CLP) was performed in male mice under anesthesia. The group pretreated with the P. acnes-killed preparation showed 80% survival at the end of the experiment (10 days) while the sub-lethal group showed 40% survival. There was an increase in the recruitment of leukocytes to the infection site in animals pretreated with the P. acnes-killed preparation, which was confirmed by a histological analysis of the cecum. Reduction in the Tumor Necrosis Factor-alpha (TNF-alpha) level was observed in the group prophylactically treated with the P. acnes-killed preparation compared to the level in the sub-lethal group. However, significant changes were not observed in Interleukin-1β (IL-1) and Interleukin-6 (IL-6) levels between the groups prophylactically treated with P. acnes and those subjected to sub-lethal sepsis. Treatment with the P. acnes-killed preparation also reduced lung injury and reduced the nitric oxide (NO) levels in the peritoneal fluid of the treated animals compared to the levels recorded in the sub-lethal group, a result probably related to the increased recruitment of neutrophils and increased survival. The results obtained suggest that prophylactic treatment P. acnes can mitigate the effects of sepsis, increasing the survival of mice.

Key words: Immunomodulation, sepsis, cellular migration, nitric oxide.

INTRODUCTION

Sepsis is defined as Systemic Inflammatory Response Syndrome (SIRS) caused by infection, mainly by bacteria but also by fungi and virus (Bone et al., 1992; Huttunen and Aittoniemi, 2011). Sepsis is characterized by an
unregulated systemic inflammatory response followed by immunosuppression (Dejager et al., 2011), and is associated with high mortality rates and high intensive care unit (ICU)-related costs (Carvalho and Trotta, 2003; Chalupka and Talmor, 2012).

Cecal ligation and puncture (CLP) is currently the most widely used animal model of sepsis (Buras et al., 2005; Deitch, 2005), showing a profile similar to that in human sepsis (Remick et al. 2000). Human sepsis is currently hypothesized to involve an initial proinflammatory burst responsible for hypotension and organ dysfunction, followed by a compensatory anti-inflammatory immune response that leads to an immunosuppressed state, often called immune depression or immune dysfunction (Hotchkiss and Karl, 2003; Riedemann et al., 2003).

In the proinflammatory phase there is involvement of neutrophils, lymphocytes, dendritic cells, macrophages, and endothelial cells, which results in an increase of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), platelet-activating factor (PAF), and reactive oxygen species (ROS) such as OH• and nitric oxide (NO) (Doi et al., 2009). TNF-α, IL-1 and IL-6 are three cytokines essentially responsible for the features of SIRS and could be potentially useful as biomarkers of sepsis. Beside these markers, interleukin-8 (IL-8), monocyte chemoattractant protein (MCP-1), interleukin-10 (IL-10), C-reactive protein (CRP), procalcitonin (PCT), and lactate can also be used as markers in sepsis. However, no single biomarker of sepsis are ideal, but many are helpful in identifying critically ill patients (Faix, 2013).

Cytokine production is stimulated by an invasion of pathogenic microorganisms, coordinating a wide range of inflammatory reactions at the tissue level, and thus playing a prominent role in the pathogenesis of sepsis (Van Der Poll, 2001). Alexander et al. (1991) showed that treatment with recombinant human TNF-α reduced mortality in CLP-induced sepsis.

ROS exert several beneficial physiologic functions, such as intracellular signaling for several cytokines and growth factors, second messengers for hormones, and redox regulation. Despite their importance as a defense mechanism against invading pathogens, a massive production of ROS or a deficit in oxidant scavengers and antioxidant defenses results in oxidative stress, a key element in the deleterious processes in sepsis (Matejovic et al. 2007; Fialkow et al. 2007). The NO plays a key role in the pathophysiology of sepsis. Benjamin et al. (2002) observed that the NO production from the inducible nitric oxide synthase (iNOS) isoform can exhibit a dual effect in sepsis: mediation of the microbicidal activity of neutrophils at the infection site; and in high reduction in rolling and adhesion of neutrophils to endothelial cells.

**Propionibacterium acnes** is a gram-positive bacillus commonly found on human skin. In mice, *P. acnes* is able to induce biological effects that modulate the innate and acquired immune responses, enhancing phagocytosis and tumoricidal activity of macrophages, as well as acting as an adjuvant in the antibody response, increasing resistance to infection (Braga et al., 2003; Mussalem et al., 2012). *P. acnes* has been widely studied due to its immunomodulatory effects when administered as an inactive microorganism in experimental models (Megid et al., 2006; Mussalem et al., 2012; Perry and Lambert, 2006; Squaiella et al., 2006). In previous studies, this microorganism has shown a number of useful activities, including antiviral, anticancer, antiparasitic, and antibacterial (Perry and Lambert, 2006) and it has an effect on lethal sepsis (Silva et al., 2013).

A previous study performed by our research group demonstrated that a **Propionibacterium acnes**-killed preparation had an important immunomodulatory role in lethal sepsis. Owing to its potent adjuvant effect on immune therapy and the lack of studies with *P. acnes* in sub-lethal sepsis, we evaluated the effect of a *P. acnes*-killed preparation in sub-lethal sepsis induced by CLP in mice.

**MATERIALS AND METHODS**

**Experimental animals**

Male mice (*Mus musculus*) weighing between 18 to 22 g were provided by the animal facilities of Federal University of Pernambuco – UFPE, Recife, Brazil. All animals were housed in a room with controlled temperature (22±2°C), humidity (50 to 60%), and a 12 h/12 h-light/dark cycle. Water and food were made available to the animals without restriction. The Animal Studies Committee of the Federal University of Pernambuco approved the experimental protocols (number 29076.036251/2013-77). The animals were treated according to the ethical principles of animal experimentation of SBCAL (Brazilian Society of Laboratory Animal Science) and the norms of the National Institute of Health Guide for Care and Use of Laboratory Animals.

**Drugs and reagents**

The *P. acnes*-killed preparation was produced by Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE), Brazil, with a concentration of 4 mg/2 ml (marketed by name Imunoparvum®); the dose used was 0.02 ml (0.04 mg/animal). Other drugs and reagents used in this study were as follows: TNF-α, IL-6, and IL-1 kits were purchased from eBioscience, San Diego, California, USA. Ceftriaxone was purchased from EMS, São Paulo, Brazil.

**Experimental design**

Polymicrobial sepsis was induced by the CLP method according to
the earlier described protocol (Rittirsch et al., 2009). The animals were anesthetized with intraperitoneal ketamine solution (50 mg/kg) and xylazine (20 mg/kg). After anesthesia, a midline laparotomy was performed with a 22 G needle, through which it was possible to expose the cecum and perform a ligation, and transverse perforation to induce sub-lethal sepsis. After surgery, the cecum was put back into its original position inside the abdomen, and the incision was closed in two layers with nylon suture 4-0. Immediately after surgery, each animal received a subcutaneous injection of 1 ml saline at 37°C as a resuscitation fluid with the purpose of preventing postoperative hypotension. For survival analysis, the animals were divided into five groups: Group 1 – Sham (n = 10); mice underwent laparotomy, but the cecum was not punctured; Group 2 – Sub-lethal group (n = 10): the mice were subjected to sepsis by CLP; Group 3 – prophylactic treatment with the *P. acnes*-killed preparation (n = 10): The animals were pretreated with the *P. acnes*-killed preparation (0.04 mg/animal) by intramuscular route on days 1, 6 and 11th day before sepsis induction. On the 12th day the animals were subjected to sepsis by CLP; Group 4 - sub-lethal sepsis and treated with ceftriaxone (n = 10): After induction of sepsis, the animals were treated with ceftriaxone by intramuscular route (i.m.), once daily for four days; and Group 5 – pretreatment with the *P. acnes*-killed preparation (1, 6 and 11th day), subjected to sub-lethal sepsis, then post-treated with ceftriaxone, once daily for four days (n = 10). For the analysis of the inflammatory parameters, the animals were divided into three groups (n = 24). Group 6 – sham (n = 8); Group 7 – sub-lethal control (n = 8): the mice were subjected to sepsis by CLP; and Group 8 - pretreated with the *P. acnes*-killed preparation, then subjected to sepsis (n = 8): The animals were pretreated (i.m.) with three doses of the *P. acnes*-killed preparation (1, 6 and 11th day), and on the 12th day the animals were subjected to CLP sepsis.

**Cellular migration**

The animals from groups 6, 7, and 8 were euthanized 24 h after induction of sepsis, and the peritoneal cavities were washed with 3 ml of PBS containing 3 mM EDTA. Peritoneal lavage was collected aseptically and stored at -40°C until further analysis. Total white blood cells (WBC) counts were performed using an automatic counter (ABX Micros 60). The differential count of the number of neutrophils in the exudate was carried out on cytocentrifuge slides stained with May-Grunwald-Giemsas, under a light microscope with 100X magnification.

**Determination of cytokines in the peritoneal lavage fluid**

For the cytokine level evaluation, the peritoneal lavage fluid was centrifuged for 10 min at 350 g, and the supernatant was stored at -40°C until the time of analysis. The concentrations of TNF-α (a sensitivity of 8 pg/mL, and a standard curve of 8-1000 pg/mL), IL-1β (a sensitivity of 8 pg/mL, and a standard curve of 8-1000 pg/mL), and IL-6 (a sensitivity of 4 pg/mL, and a standard curve of 5 to 500 pg/mL) were determined using ELISA according to the manufacturer’s instructions (eBioscience, San Diego, California, USA).

**Quantification of nitric oxide in the peritoneal lavage fluid**

The nitrite concentration in the peritoneal lavage fluid was used as an index of nitric oxide production by the Griess’ reaction. Briefly, 50 µL of each sample and 50 µL of Griess reagent were placed in a 96-well microtiter plate and incubated at room temperature and protected from light for 10 min. The absorbance was measured using a wavelength of 560 nm in a microplate reader and the nitrite concentration was determined by comparing the sample absorbance to a standard curve for sodium nitrite. The results were performed in triplicate and expressed in µM (Giustarini et al., 2008).

**Histopathological analyses**

To evaluate neutrophil migration in the cecum wall and lung of mice with sepsis, the animals of the sham group and those pretreated with *P. acnes* were euthanized 24 h after the induction of sepsis by CLP. Fragments of the cecum and lung were removed, fixed in 10% formalin for 24 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Fragments of 5 µm were stained with hematoxylin-eosin for histopathological analysis of the inflammatory response.

**Immunohistochemical evaluation of iNOS in lung tissue**

Lung tissue sections of the sham, sub-lethal, and treated groups were cut and adhered to slides treated with 3-amino-propyltriethoxysilane (APES (Sigma, USA)). Briefly, the samples were rehydrated in ethanol (70 to 100%) after deparaffinization with xylene. To minimize endogenous peroxidase activity, the slides were treated with 10% (v/v) H2O2 in water for 15 min. The sections were washed with 0.01 M PBS (pH 7.2) and blocked with 1% BSA, 0.2% Tween 20 in PBS for 1 h at room temperature. The sections were incubated overnight at 4°C with anti-iNOS (Abcam, CA, USA, 1: 50). The chromogen 3,3-diaminobenzidine was used to visualize the antigen-antibody reaction with avidin-biotin peroxidase (Dako Universal LSAB + Kit, Peroxidase). The slides were counterstained with hematoxylin. Positive staining resulted in a brown reaction product. Five pictures at the same magnification were analyzed quantitatively using the Gimp 2.6 software program (GNU Image Manipulation Program, UNIX platforms) (Ribeiro et al., 2014).

**Statistical analysis**

The survival of mice was expressed as a percentage of surviving animals analyzed by the Mantel-Cox test and differences were considered significant at p < 0.05. All other results were expressed as the mean ± standard deviation. Statistical analysis was performed by a one-way analysis of variance using ANOVA followed by a Tukey’s test, with a significance level of 0.05, using the GraphPad software version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

**RESULTS**

**Effect of the *P. acnes*-killed preparation on the survival of animals subjected to sub-lethal sepsis by CLP**

Survival was assessed every 12 h after CLP during the 10 day period. The survival of animals in the sub-lethal group five days after the induction of sepsis was 40% and remained so until the last day of the observation (Figure 1). A reduction in mortality was observed in the animals pretreated with the *P. acnes*-killed preparation, showing 80% survival after 10 days of observation. The same result was observed in the animals post-treated with ceftriaxone (standard drug). To assess whether *P. acnes* could act synergistically with ceftriaxone, an associative
group using *P. acnes* + ceftriaxone as post-treatment was assessed and there was a survival rate of 100%, a result similar to that obtained in the sham group.

**Cell migration**

A significant increase in the total number of leukocytes was observed in the peritoneal cavity of the group pretreated with the *P. acnes*-killed preparation (18.54±0.88 x 10³ cells/mm³), compared to the sub-lethal group (12.48±2.06 x 10³ cells/mm³) (Figure 2A). A significant reduction in neutrophil migration to the peritoneal cavity was observed in the animals of the sub-lethal group, a characteristic of sepsis (Figure 2B).

**Effect of the *P. acnes*-killed preparation on levels of TNF-α, IL-1β, and IL-6**

Figure 3 (A-C) shows the concentrations of TNF-α, IL-1β, and IL-6 in the peritoneal lavage fluid of the groups treated with the *P. acnes*-killed preparation, sham, and sub-lethal 24 h after surgery. A significant reduction in TNF-α levels in the pretreated group was observed when compared to the sub-lethal group; however, no changes were observed in IL-1β and IL-6.

**Quantification of nitric oxide in the peritoneal lavage fluid**

24 h after induction of sepsis by CLP, the peritoneal lavage fluid samples were collected for measurement of nitric oxide (NO). The group treated with the *P. acnes*-killed preparation showed a significant reduction in the NO level when compared to the sub-lethal group (Figure 4).

**Evaluation of the inflammatory infiltrate in the cecum**

The group pretreated with the *P. acnes*-killed preparation showed an intense neutrophilic infiltrate (Figure 5C) in the cecum when compared with the sub-lethal group, which showed moderate infiltrate (Figure 5B).

**Histopathology of the lung tissue**

The sub-lethal group showed a reduction in alveolar lumen, and exacerbated presence of bleeding and inflammatory cells, results consistent with the induction of acute lung injury found in sepsis (Figure 6B). On the other hand, the group pretreated with the *P. acnes*-killed preparation showed better aspect of the morphology of the lung parenchyma when compared to the sub-lethal group (Figure 6C). Histological analysis of the sham group showed preserved alveoli and bronchioles, with integrity of the septum (Figure 6A).

**Immunohistochemical evaluation of iNOS in lung tissue**

An immunohistochemical analysis of the lung tissue showed a significant reduction in iNOS levels in the group pretreated with the *P. acnes*-killed preparation when compared to the levels of the sub-lethal group. The results are shown in Figures 7 and 8.

**DISCUSSION**

The results showed that pretreatment with *P. acnes*-killed decreased the mortality rate and attenuated acute lung injury induced by CLP by regulating the levels of NO, and recruiting neutrophils to the infection site. Neutrophils...
Figure 2. Total (A) and differential (B) counts of leukocytes in the peritoneal lavage fluid. Total and differential cell counts were evaluated 24 h after cecal ligation and puncture (CLP). The results were expressed as mean ± S.D. **P < 0.01, ### P < 0.001 compared to the sham group, *** P < 0.001 compared to sub-lethal group using Tukey’s post-test.

play an important role in the innate immune response (Summers et al., 2010), because they are the first cells to arrive at the site of injury or infection, and thus serve as the first line of defense of the body, playing an important role in the control of fungal and bacterial infections (Drescher and Bai, 2013; Kumar and Sharma, 2010). The failure of neutrophils to migrate to the focus of infection in sepsis is associated with the difficulty of controlling the infection, increased bacterial spread, and high mortality (Benjamin et al., 2000; Maciel et al., 2008).

In this study, this failure was observed in the sub-lethal group, which presented difficulty in eradicating the infection. However, the pretreatment with _P. acnes_ improved the animals’ survival through induction recruitment of total leukocytes, particularly of neutrophils, to the initial focus of infection. These results were confirmed by histopathological examination of the cecum. Previous studies by the study group using microorganisms in the treatment of sepsis induced by CLP also noted an improvement in the survival of animals, associated with an increased recruitment of leukocytes and neutrophils (Campos et al., 2013; Silva et al., 2013).

The neutrophil response to invading pathogens occurs because of the ability to store cytotoxic granules enriched with different antimicrobial molecules: cationic peptides, protease, myeloperoxidase, and lactoferrin (Kumar and Sharma, 2010). In addition, there is the production of reactive oxygen species (ROS) acting in an attempt to destroy microorganisms invading the host (Drescher and Bai, 2013). In sepsis, the failure of neutrophil migration is related in part to the high release of nitric oxide (Benjamim et al., 2000). Nitric oxide is an important mediator involved in sepsis and its elevation is assigned a high expression of the inducible isoform of NO synthase (iNOS) (Araujo et al., 2012; Mansart et al., 2003; Tracey et al., 1995).

The study observed a high production of NO metabolites (nitrite) in the peritoneal cavity of the sub-lethal group, while in the group pretreated with _P. acnes_ there was a significant inhibition of nitrite production after...
Figure 3. Effect of prophylactic treatment with the *P. acnes*-killed preparation on TNF-α (A), IL-1β (B), and IL-6 (C) levels in the peritoneal cavity of mice (*n* = 6) subjected to cecal ligation and puncture (CLP). The cytokine levels in peritoneal exudates were determined at 24 h after surgery in sham, sub-lethal sepsis, and the prophylactic treatment with *P. acnes*-killed groups. The results were expressed as mean ± SD. 🌈 🌈 🌈 *P* < 0.001 compared to the sham group, *P* < 0.05 compared to sub-lethal sepsis using Tukey’s post-test.

CLP. Thus, it is possible to assume that *P. acnes* may inhibit NO production, enhance neutrophil migration ability to the infection focus and, consequently, improve the survival rate of animals subjected to sepsis.

The increased production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 has been implicated in the
Figure 4. Determination of nitric oxide (NO) in the peritoneal lavage fluid of animals prophylactically treated with the P. acnes-killed preparation (n = 5) and subjected to sub-lethal sepsis by CLP. The results were expressed as mean ± SD. ** P < 0.01, *** P < 0.001 compared to the sham group. *** P < 0.05 compared to sub-lethal sepsis using Tukey’s post-test.

Figure 5. Photomicrograph showing migration of inflammatory cells in the cecum of mice subjected to prophylactic treatment with the P. acnes-killed preparation. HE staining. 400x. Sham group (A), sub-lethal sepsis (B) and prophylactic treated with P. acnes-killed (C).

development of sepsis, and contributes to tissue damage and increased inflammatory response (Cao et al., 2012; Li et al., 2013; Yang et al., 2009; Zou et al., 2013). TNF-α, a pro-inflammatory cytokine, is considered an important mediator in inflammation, and is present in the serum of animals and humans undergoing sepsis (O’Callaghan and Redmond, 2006). Studies conducted by Silva et al. (2013), evaluated the effect of P. acnes-killed in severe sepsis, and observed a reduction in TNF-α levels and a consequent increase in survival. Similar results were reported by Campos et al. (2013), who observed that the increase in the survival rate in mice
**Figure 6.** Photomicrograph of histopathological changes in the lung tissue of mice subjected to pretreatment of *P. acnes*-killed preparation and subjected to sepsis by CLP. HE staining. 400x. Sham group (A), sub-lethal group (B), and pretreated with the *P. acnes*-killed preparation (C).

**Figure 7.** Immunohistochemical analysis of iNOS in lung tissue of animals subjected to sepsis by CLP. Sham group (A), sub-lethal sepsis (B) and prophylactic treatment with *P. acnes* preparation (C) 400x.
subjected to sepsis and pretreated with *Zymomonas mobilis* was associated with the reduction in TNF-α levels. These results corroborated with that of this study, where it was observed that *P. acnes* also significantly reduced TNF-α levels in peritoneal lavage fluid 24 h after CLP. However, it did not reduce IL-1β and IL-6 levels when compared to the sub-lethal group.

To better understand the mechanism by which *P. acnes* is capable of mediating protection against polymicrobial sepsis, the study investigated its effect on acute lung injury. After installation of sepsis, the lung is often the most affected organ during the early development of multiple organ dysfunction syndrome (Andrews et al., 2005; Xu et al., 2013). The increased expression of NO from the inducible isoform (iNOS) in lung tissue in animal models and humans is associated with Acute Lung Injury (ALI) and increased mortality in sepsis (Shelton et al., 2008). Neutrophils are also appointed as mediator cells of acute lung injury and are related to acute respiratory stress syndrome (Brown et al., 2006).

A reduction in iNOS expression in lung tissue was observed in the group treated with *P. acnes*-killed compared to the sub-lethal group, suggesting that a decrease of NO can contribute beneficially to the survival of animals submitted to CLP. Histological analysis revealed that the sub-lethal group showed reduced alveolar lumen, in addition to the heightened presence of inflammatory cells and hemorrhage. The group pretreated with *P. acnes* showed improvement of the inflammation, differing only by the presence of mild leukocyte infiltration, with consequent improvement in survival. Similarly, Li et al. (2013) observed a reduction in levels of NO in the lung, and consequent improvement in survival of animals subjected to sepsis. Several studies have reported that an increase in survival of animals subjected to sepsis by CLP was associated with the attenuation of inflammatory cell infiltration and a reduction of injury in lung tissue (Campos et al., 2013; Li et al., 2013; Xu et al., 2013; Zou et al., 2013).

**Conclusion**

In summary, the study results show that *P. acnes* presents beneficial effects in sepsis by attenuating the inflammatory response, reducing acute lung injury, and improving the recruitment of neutrophils to the infection site. The mechanism appears to involve its ability to inhibit inflammatory response via modulating nitric oxide and TNF-α levels. These findings would suggest that administration of the *P. acnes*-killed preparation may act as an alternative therapeutic strategy beyond antibiotic treatment.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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