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ARTICLE

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Preparation of sertraline-loaded chitosan nanoparticles and the pharmacokinetics studies

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Sertraline is a worldwide used antidepressant for perinatal depression. However, the secreting of sertraline into milk aroused the concerns for the potentially negative effects on breastfed infants. The present study aims to alter the biodistribution of sertraline by loading the drug into chitosan-nanoparticles. Our results demonstrated an effective way to load the water-soluble sertraline into nanoparticles, and keep the size around 200 nm. The in vivo results demonstrated the difference of sertraline concentration in plasma after the intravenous injection through the marginal ear vein in rabbits. Because of the protection of nanoparticles, the drug concentration in plasma increased 5 h post the injection, which may delay the tissue-distribution of sertraline. Thus, our results indicate that the nanoparticles-encapsulation may change the biodistribution of sertraline and offer a safe window for breastfeeding. Nevertheless, the sertraline in breast milk was still detectable after injection of sertraline-loaded nanoparticles. Thus, it still needs caution for lactating women to have antidepressant drugs during breastfeeding.

Key words: Sertraline, chitosan, nanoparticles, pharmacokinetics.

INTRODUCTION

Perinatal depression is an increasingly common psychiatric disease for those women who are in pregnancy and postpartum and antidepressants are often used during these periods, (Cooper and Murray, 1995; Evans et al., 2001; Marcus et al., 2003; Gaynes et al., 2005; Dietz et al., 2007; Patil et al., 2011). Therefore, the administration of antidepressant drugs while breastfeeding is of great concern to both mothers and physicians, because this requires the knowledge of the extent to which drugs are excreted into breast milk. Most antidepressant drugs pass into breast milk to some extent through passive diffusion (Begg, 2006).

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Sertraline (Zolof) is one of the members belonging to the
group of selective serotonin reuptake inhibitors (SSRIs) in brain (Rao et al., 2009). It was introduced
firstly in 1988 (Doogan and Caillard, 1988) and noted to be the most prescribed antidepressant with no adverse
effects (Whitby and Smith, 2005). Because of some advantages, like an improved tolerability and adverse
effect profile and relative safety in over dosage (Doogan and Caillard, 1988; Henry, 1991; Grimsley and, 1992),
SSRIs have been one of the first line choices for the treatment of depression (Wen and Walker, 2004; Field,
2008). However, it has been reported that sertraline will be secreted to milk when administrated by breastfeeding
mother (Alshuler et al., 1995; Stowe et al., 1997; Kristensen et al., 1998; Wisner et al., 1998), which may
cause potentially negative effects of antidepressants on breastfed infants. Stowe et al. (1997) found that sertraline
and desmethylsertraline are present in the breast milk of nursing. Detectable concentrations of sertraline are found
in three of eleven nursing infants. Moreover, in another case (N=15 women and 182 breast milk samples), the
highest concentrations are observed in the hindmilk 8 to 9 h after maternal ingestion of sertraline (Stowe et al.,
2003). In addition, the long-term neurobehavioral influence of this antidepressant drug on these infants is
more worried. Thus, most postpartum depressed mothers will not breastfeed or reduce the breastfeeding periods
of time, as they concern about the potential negative effects of antidepressants on their breast milk and, in turn, on
their infants’ development (Field et al., 2002; Field, 2008). Therefore, how to avoid or decrease the antidepressants
in breast milk has been a challenge for the treatment to perinatal depression.

 Nanoparticles are, in general, colloidal particles, less
than 1000 nm, that can be used for better drug delivery
and prepared either by encapsulating the drug within a
vesicle and or by dispersing the drug molecules within a
matrix (Saha et al., 2010). Because of the unique
physicochemical properties of nanoparticles, nanoparticles based drugs may have improved solubility,
pharmacokinetics, and biodistribution as compared to small molecule drugs (Dobrovolskaia et al., 2008). They
can alter and improve the biodistribution, pharmacokinetic and pharmacodynamics properties of various types of
drug molecules (Vasanthakumar et al., 2010). Furthermore, nanoparticles based drugs can often extend the
circulation times in plasma than small molecule drugs, which cause their longer half-lives. Thus, nanoparticulate seems to have the advantages in altering the biodistribution and extending the half-lives of drug
molecules.

In the present study, sertraline was used as a model
drug to prepare the sertraline-loaded nanoparticles, and
compared the biodistribution of sertraline nanoparticles in
rabbits’ plasma with that of the sertraline solution to
investigate whether nanoparticles could change the
biodistribution profile of sertraline. Here, chitosan is
chosen as the material for drug carrier, because it has
great potential as a biomaterial for the construction of
nanosized drug, and is generally regarded as non-toxic,
biocompatible and biodegradable material (Knapczyk,
1989; Chandy and Sharma, 1990; Hirano et al., 1990;
Bersch et al., 1995). The sertraline-loaded chitosan
nanoparticles were successfully prepared based on ionic
gelation of chitosan with tripolyphosphate (TPP) anions.
Many groups have reported the pharmacokinetics of
sertraline molecules in breast milk, mother’s serum and
infant’s serum, but the distribution of chitosan
nanoparticles encapsulated sertraline have not been
reported yet to our knowledge.

Our results demonstrated that chitosan nanoparticles
could prolong the residence time of sertraline in circulation
when compared with its solution form, and might offer a
safe window for breastfeeding. Nevertheless, there was
still some sertraline secreted into the breast milk after
injection of sertraline-loaded nanoparticles. Thus, it still
needs caution for lactating women to have antidepressant
drugs during the breastfeeding.

MATERIALS AND METHODS

Reagents and animals

Sertraline hydrochloride was bought from WuHan Weishunda
Technology Development Ltd. (China). HPLC-grade acetonitrile and
methanol were obtained from Fisher Scientific (USA). 95% n-
hexane, acetic acid, potassium dihydrogen phosphate, sodium
hydroxide crystals and 1M hydrochloric acid, all of analytical
reagent (AR) grade were obtained from Sinopharm Chemical
Reagent Co., Ltd. (China). Chitosan was obtained from Yuhuan
Ocean Biochemical Co., Ltd. (China), Sodium TPP was bought from
Shijiazhuang Shinearly Chemicals Co., Ltd. (China). Milli-Q water
was used to prepare the buffers and aqueous solutions.

Pregnant New Zealand White rabbits (Body weight 3.0 to 4.0 kg)
were purchased from Zhejiang University of Traditional Chinese
Medicine. All procedures involving animals were approved by the
Institution Animal Care and Use Committee of this center. Animals
were acclimatized in our laboratory 7 days before delivery. During
this period, animals were housed separately in cages with
controlled light cycle (12/12 h).

Preparation of sertraline-loaded nanoparticles

The sertraline-loaded nanoparticles were prepared by a crosslinking
way, which the free amino groups of chitosan would crosslink to the
negative ion of TPP to form globular gel. In general, chitosan
solution 2.5 mg/ml was prepared by dissolving the polymer in 1%
(v/v) acetic acid aqueous solution and stirred for 10 min. Then, 50
mg sertraline was added into the solution and stirred until the
sertraline was absolutely dissolved. After that, the pH of the solution
was adjusted to 5.0 to 6.0 using 1 mol/L NaOH. The chitosan
solution was further stirred for 0.5 h at room temperature. Finally,
sodium TPP, the counter ion, was dissolved in pure water to
prepare a 1 mg/ml solution, and added to the chitosan solution under
mild magnetic stirring to form chitosan nanoparticles. The
nanoparticles solution was centrifuged at 18,000 rpm and 4°C for
30 min and the nanoparticles at the bottom were then collected,
extensively washed with water to remove the TPP and the acetic
acid, and lyophilized at last.
Morphological characterization of nanoparticles

Transmission electron microscopy (TEM) was performed for the morphological examination of nanoparticles. The nanoparticles were stained with 2% (w/v) phosphotungstic acid aqueous solution for 10 s, immobilized on copper grids with formvar and were dried overnight before microscopy.

The particle size and polydispersity index (PDI) of nanoparticles were measured by Nano-S90 laser particles size analyzer (Zetasizer Nano-S90, Malvern, UK) after dilution of nanoparticles suspension with distilled water (30 fold).

Determination of entrapment efficiency of sertraline

The quantity of sertraline entrapped into the nanoparticles was calculated by the ultracentrifugation method. In detail, the nanoparticles were centrifuged at 3000 rpm for 10 min to separate the nanoparticles. The resulting supernatant was transferred to ultracentrifuge tubes (Beckmann Instruments, Fullerton, CA) and further centrifuged at 20000 rpm for 30 min. The sediment obtained was re-suspended in double distilled water (DDW, 10 ml) with the aid of a sonicator and centrifuged at 20000 rpm for 30 min. This process (that is, re-suspension of sediment and centrifugation) was repeated three times and all of the supernatants were collected. The concentrations of sertraline in supernatants were measured by high performance liquid chromatography (HPLC) method.

The entrapment efficiency of the sertraline nanoparticles was calculated as: entrapment efficiency (%) = (total sertraline−free sertraline)/total sertraline.

HPLC analysis

**Chromatographic conditions**

A modified HPLC–UV method described by Dodd et al. (2000) was used for the quantification of sertraline in various samples. The HPLC system consisted of an Agilent series 1200 Chemstation, Agilent 1200 VWD absorbance detector (Agilent Technologies Singapore (International) Pte. Ltd., USA). Sample separation was performed on a Diamons® C18 (150 × 4.6 mm, 5 μm) (Dikma, China) with a guard column (EasyGuard® C18, 10 × 4.0 mm) (Dikma, China). The mobile phase consisting of phosphate solution (15 mM, pH=3.0); Acetonitrile (60:40, v/v) was prepared daily and filtered through a Millipore membrane filter (0.22 μm) and degassed by sonication in an ultrasonic bath before use. The flow rate and the detection wavelength for monitoring the eluents were 1 ml/min and 225 nm, respectively. The analysis was carried out at 30°C. The injection volume was 20 μl. Data collection and processing were performed using Agilent series 1200 Chemstation software.

**Preparation of stock and standard solutions**

Stock solutions containing 1 mg/ml of individual sertraline were prepared in water. Working standards (100 μg/ml) were prepared by dilution of individual aliquots of stock solution with the same solvent. The solutions were stable at least for 1 month at 4°C. Appropriate dilutions of the individual working solutions of sertraline were made and used for constructing the calibration curves.

**Extraction procedure for calibration of plasma specimens**

Calibrations for sertraline in plasma were done by adding appropriate volumes of working standard (100 μg/ml) solution of sertraline to 90 μl of plasma in 1.5 ml EP tubes to give a range of sertraline concentrations ranging from 1 to 40 μg/ml. 100 μl of 1M NaOH was added to the specimens and vortexed for 15 s. 1 ml of n-hexane and ethyl acetate mixture solution (v/v, 4/1) was then pipetted into each of the specimen tube and vortexed for 2 min and shook on an orbiter shaker (QB-600, Kylin-Bell Lab Instruments Co., Ltd., China) for 15 min. After that, the specimens were centrifuged at 3500 rpm for 15 min. 0.9 ml of the organic layer was then transferred to clean EP tubes and dried using a stream of nitrogen gas at 37°C. At last, the specimens were dissolved in 200 μl of the mobile phase for HPLC analysis.

**Extraction procedure for calibration of milk specimens**

Calibrations for sertraline in milk were done by adding appropriate volumes of working standard (100 μg/ml) solution of sertraline to 90 μl of milk in 1.5 ml Eppendorf (EP) tubes to give a range of sertraline concentrations ranging from 1 to 20 μg/ml. The followed procedures were similar as mentioned earlier.

**Plasma and milk distribution study**

Milk and blood sampling was carried out from rabbit during lactating. Sertraline (30 mg/kg) was administered via marginal ear vein. Serial blood samples (0.5 ml) were drawn into heparinized syringe before starting the kinetic study at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h after drug administration and were always replaced with an equal volume of saline solution. Serial milk samples were also taken at different time points after drug administration by manual expression into a centrifuge tube connected to a vacuum system. At each time, the gland was emptied as completely as possible. The blood and milk samples were stored frozen at -20°C until analysis. The extraction procedure of plasma and milk specimens were the same as mentioned earlier. Sertraline in various samples was quantified by HPLC-UV method.

**Statistical analysis**

Statistical evaluation of differences experimental group means was analyzed by multiple Student t-tests. Data were expressed as the mean ± standard deviation (SD). Data points were from at least three independent experiments.

**RESULTS**

**Preparation and characterization of sertraline nanoparticles**

In this study, a serial of mass ratios of chitosan (CS)/TPP was set and the particle size, PDI and entrapment efficiency was detected, respectively to investigate the best prescription for sertraline-loaded chitosan nanoparticles. Results are shown in Table 1 and Figure 1a. When the mass ratios of CS/TPP were from 8 to 16, the diameter of the prepared nanoparticles was around 200 nm and with the relative low PDI. Although, quite good quality of nanoparticles at mass ratios of CS/TPP was also acquired from 5 to 8; the size of the nanoparticles seems a little large that it may not suit for the intravenous injection. Our former study had reported that the CS/TPP ratio had influence on the stability of the nanoparticles (Li et al., 2010). The stability will be decreased with the
increasing amount of TPP. Here, the nanoparticles, were relative stable below the ratio of 10. In addition, when the mass ratio is at 10, the highest sertraline entrapment efficiency with 15.60% was acquired. Thus, the sertraline-loaded chitosan nanoparticles prepared at mass ratio of 10 were studied in the following experiments. The transmission electron microscopy (TEM) micrographs of the sertraline nanoparticles with the mass ratio of CS/TPP at 10 were demonstrated as shown in Figure 1b. These nanoparticles were generally spherical, and the particle size distribution was in an acceptable range. The mean diameter was 198±29 nm and the PDI was 0.253 (n=3). Figure 1c demonstrated the size distribution of the nanoparticles obtained by the Zetasizer.

**HPLC assay of sertraline in plasma and milk**

The calibration curves of sertraline in blank plasma and milk were \( A_p = 20.16 \times C_p - 32.622 \) (where \( A_p \) represents peak area, \( C_p \) represents the concentration of sertraline in plasma, \( R^2 = 0.9998 \)) and \( A_m = 34.422 \times C_m + 50.409 \) (\( A_m \) represent peak area, \( C_m \) represents the concentration of sertraline in milk, \( R^2 = 0.9998 \)), respectively. The retention times of sertraline in plasma and milk were 5.93±0.21 and 6.20 ± 0.18 min, respectively. It was believed from these results that this analysis method was accurate and precise with coefficients of variation with intra-and inter-day relative standard deviation (RSD) below 10% for all the biological samples. The precision and the accuracy of this method were determined by adding known amounts of sertraline to blank plasma and milk and the results conformed to a certain standard within 85.0 to 110.0%. The limit of quantification (LOQ) in plasma was 0.02 μg/ml.

**Plasma pharmacokinetics study**

After a single intravenous administration of sertraline solution or sertraline nanoparticles in rabbits at a dose of 30 mg/kg, the plasma concentrations of sertraline were assessed. Figure 2 compared the plasma drug concentration-time curves of sertraline solution and sertraline nanoparticles. The main pharmacokinetic parameters were derived by the software of Data Access System 2.0 version (DAS, Mathematical Pharmacology Professional Committee of China, Shanghai, China) and summarized in Table 2. According to the analysis of models and parameters, a two-compartment model presented the best fit to the plasma drug concentration time curves obtained in rabbits. The biological half-lives of sertraline injection and sertraline nanoparticles in the phase of eliminate were 15.269 and 43.565 h, respectively. And the half-lives in the phase of distribution were 0.242 and 43.534 h, respectively. The mean area under the plasma concentration-time curve from zero to infinity \( \left( \text{AUC}_{(0-\infty)} \right) \) of sertraline nanoparticles (453.245 μg*h/ml) was about 4 times greater than that of the sertraline solutions (105.126 μg*h/ml). The possible reason for the aforementioned results is mainly due to the protection effect of the chitosan nanoparticles. This is because the encapsulated sertraline needs to be released from the nanoparticles in the circulation firstly. And then distributed to the tissues and be eliminated like the sertraline solution. The increased drug concentrations in plasma of sertraline nanoparticles from 4 to 12 h (Figure 2) may be due to the release of sertraline from the nanoparticles.

**Concentrations of sertraline nanoparticles in rabbits’ milk**

The concentrations of sertraline-loaded nanoparticles in lactation rabbits’ hindmilk were also investigated to check whether the sertraline was detectable in milk. The samples were taken after a single intravenous administration of sertraline nanoparticles in lactation rabbits. Figure 3 demonstrated the drug concentrations of sertraline nanoparticles in milk. It was believed that the drug concentrations of sertraline-loaded nanoparticles were detectable at 1, 2, and 3 h in rabbits’ milk after i.v. administration of sertraline nanoparticles using the analysis method established in this study.

**DISCUSSION**

Dilemmas about whether or not to contraindicate breastfeeding arise most commonly in relation to postpartum depression as antidepressants took by the nursing

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**Table 1. Size and size distribution of sertraline nanoparticles with different mass ratios of CS/TPP.**

<table>
<thead>
<tr>
<th>Mass ratio (CS/TPP)</th>
<th>Diameter (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.7</td>
<td>240±4</td>
<td>0.077</td>
</tr>
<tr>
<td>12.5</td>
<td>217±22</td>
<td>0.230</td>
</tr>
<tr>
<td>10</td>
<td>198±29</td>
<td>0.253</td>
</tr>
<tr>
<td>8.4</td>
<td>234±6</td>
<td>0.134</td>
</tr>
<tr>
<td>6.3</td>
<td>298±28</td>
<td>0.202</td>
</tr>
<tr>
<td>5</td>
<td>313±10</td>
<td>0.139</td>
</tr>
</tbody>
</table>

**Figure 1a** shows the size distribution of the nanoparticles obtained by the Zetasizer.
Figure 1. (a) Entrapment efficiency of sertraline-loaded nanoparticles with different mass ratios of CS/TPP. When the mass ratio of CS/TPP equaled to 10, the nanoparticles had the highest entrapment efficiency with 15.6±2.1%. (b) Transmission electron microscopy (TEM) micrograph of chitosan nanoparticles loaded with sertraline (the mass ratio of CS/TPP is 10). (c) The size distributions of the sertraline nanoparticles (the mass ratio of CS/TPP is 10).
mother will distribute into breast milk (Stowe et al., 1997; Yoshida et al., 1999). As such, the nursing infants will be exposed to the drugs. Nanoparticles as thought can alter and improve pharmacokinetics and biodistribution of drug molecules contributing to the unique physicochemical properties of nanoparticles. Therefore, we tried to prepare a sertraline-loaded nanoparticle to alter the pharmacokinetics behavior and decrease the diffusion of sertraline to breast milk. Our studies demonstrated that the encapsulation of sertraline into the chitosan nanoparticles prolonged the drug residence time in plasma, as well as the area under curve (AUC). These results indicated that the nanoparticles can maintain more drug in the circulation and prevent the rapid tissue-distribution when compared with the solution. It is believed that by maintaining a high drug concentration in circulation may help decrease the secretion of sertraline into milk. As demonstrated in the plasma drug concentration-time curves, the concentration of sertraline in plasma rapidly decreased during the first 1 h, and then the concentration declined slowly. Whereas, for sertraline-loaded nanoparticles, the concentration in plasma maintained at a lower level during the first 4 h, which is because sertraline was still encapsulated in nanoparticles. Then, the concentration reached the peak at 8 h, which may due to the sustained release of sertraline from nanoparticles. And then the drug concentration decreased after 12 h. Thus, it is believed the nanoparticles can create a 4 h window for breastfeeding after the administration of antidepressants. Since most of the sertraline was still encapsulated in the nanoparticles during this period after the drug administration. Nevertheless, there was still some sertraline released from the nanoparticles in the first 4 h and could be detected in the breast milk. Therefore, it still needs caution for lactating women to have antidepressant drugs during breastfeeding. And more detail studies for the pharmacokinetics in milk are required.

Table 2. The comparative pharmacokinetic parameters after i.v. administration of sertraline and sertraline-loaded nanoparticles in rabbit (n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>C_{max} (μg/ml)</th>
<th>AUC_{(0→∞)} (μg*h/ml)</th>
<th>CL (ml/(kg*h))</th>
<th>V (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>11.382</td>
<td>105.126</td>
<td>0.19</td>
<td>1.112</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>22.434</td>
<td>453.245</td>
<td>0.044</td>
<td>2.674</td>
</tr>
</tbody>
</table>
Conclusion

The mass ratio of CS/TPP had an influence on the diameter and entrapment efficiency of the sertraline-loaded nanoparticles. It showed the best size and entrapment efficiency at the ratio of 10. These chitosan nanoparticles carrier could prolong the resistant time of sertraline in plasma and enhance AUC_{0-∞} compared with sertraline solution. This may create a 4 h window for breastfeeding. Nevertheless, the sertraline-loaded nanoparticles should still be used with caution during lactation, as sertraline in milk was still detectable after administrating with the nanoparticles.

Conflict of interest

The authors have not declared any conflict of interest.

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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 microbial 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; Sqaïella 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 23076.036251/2013-77). 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–Grünwald–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 nitrate 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 Graph Pad 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 10^3 cells/mm^3), compared to the sub-lethal group (12.48±2.06 x 10^3 cells/mm^3) (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
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

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**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.**
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 4](image)

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).

![Figure 5](image)

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 \textit{P. acnes}-killed preparation and subjected to sepsis by CLP. HE staining. 400x. Sham group (A), sub-lethal group (B), and pretreated with the \textit{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 \textit{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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