COPAIBA OIL EFFECTS ON SURVIVAL RATE AFTER CECAL LIGATION AND PUNCTURE IN MICE[1]

EFEITOS DO ÓLEO DE COPAIBA NA SOBREVIDA DE CAMUNDONGOS SUBMETIDOS À LIGADURA E PERFURAÇÃO CECAL

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SUMMARY

Objective: to assess survival rate of mice subjected to cecal ligation and puncture (CLP) treated with copaiba oil subcutaneous injection. **Methods:** twenty-one BALB/c male mice were randomly distributed into three following groups: Sham (subjected to all surgical procedures except for the CLP procedures); Control (subjected to CLP procedures with no treatment); and Experimental (subjected to CLP procedures treated with copaiba oil, 0.63 ml per kg, injected once a day subcutaneously for five days). Sepsis was induced by CLP procedure and after that, mice were monitored every 6 h till 120 h completed. Survival rate was assessed by the Kaplan-Meier curve and the long-rank test. **Results:** there was a significant difference on survival of mice subjected to copaiba oil injection ($p < 0.0001$), survival rate in Control was 0% after 72 h, as expected, but Experimental survival rate was 71.43% till the last observation time (120 h). **Conclusion:** copaiba oil treatment improved survival in mice subjected to CLP.

Keywords: Medicinal Plants; Mice; Sepsis; Survival Analysis.

INTRODUCTION

Sepsis, systemic inflammatory response syndrome and associated multiple organ failure as well as the multiple organ dysfunction syndrome remain the most common causes for fatalities during the clinical course following trauma or major surgery.[1,2]

Despite extensive research in the past, the pathophysiology of sepsis in humans is still poorly understood, and hospitalization and mortality rates of septic patients have significantly increased in the United States between 1993 and 2003.[3] In Brazil, sepsis, severe sepsis and septic shock lethality observed in intensive care units, was 16.7%, 34.4% and 65.3%, respectively.[4]

Due its high mortality rate in front of standard treatments, many efforts have been done in order to elucidate the host misbalance in response of sepsis.[3,5]

To study the underlying mechanisms of sepsis and the associated systemic inflammatory response, several experimental animal models have been developed, all of which attempt to mimic pathophysiological changes typically seen in septic patients. Cecal ligation and puncture in rodents has become the most widely used model for experimental sepsis and is currently considered as the gold standard in sepsis research.[6,7,8]

Therefore, it is urgent for searching potential therapeutic drugs to improve the survival of septic injury.[9] There are few studies about the sepsis treatment with medicinal plants,[10,11] and there was not founded any study involving the sepsis treatment with copaiba oil. It has been reported the activity of the copaiba oils against Gram-positive bacteria *in vitro*, copaiba oils obtained from *C. martii*, *C. officinalis* and *C. reticulata* exhibited good antibacterial activity against Gram-positive bacteria, including MRSA.[12]

These findings suggest that copaiba oil is a potential alternative to development of new selected agents for severe infectious disease treatment, such as sepsis.

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Therefore, the aim of this research was to assess survival rate of mice subjected to cecal ligation and puncture treated with copaiba oil subcutaneous injection.

METHODS

Ethics

The use of laboratory animals followed the Council for International Organization of Medical Sciences ethical code for animal experimentation and the principles of the Brazilian College on Animal Experimentation. The research was approved by the Ethics Committee of State University of Pará (UEPA), process number 162/08 and received financial source from National Council of Scientific and Technologic Development (CNPq), as case number 2496/09.

Sample

Twenty-one BALB/c male mice (*Mus musculus*), weighing 25-30 g were used in this research. They were stored in cages in a temperature-controlled room (22° C) with 12-h light and dark cycles, with offered water and food *ad libitum*. The mice were randomly distributed into three following groups:

Sham (SG): 7 mice, subjected to all surgical procedures except for the CLP procedures;

Control (CG): 7 mice, subjected to CLP procedures with no treatment;

Experimental (EG): 7 mice, subjected to CLP procedures with copaiba oil injection.

Substances

Copaifera reticulata Ducke oil (0.63 ml per kg) was injected once a day subcutaneously for five days in EG mice. Copaiba oil (*C. reticulata* Ducke) used were extracted and provided by EMBRAPA in the form of crude oil. The physical-chemical oil analysis and spectrophotometry was made by the Laboratory of Chemistry, Federal University of Pará (UFPA).

Preoperative setup and anesthetics

All animals were weighed to determine the amount of anesthetics to be used. The animals were anesthetized with ketamine (100 mg per kg body weight i.p.) and xylazine (10 mg per kg body weight i.p.). The intensity of anesthesia was monitored by toe pinch using tweezers, adequate anesthesia should result in no response of extremity. Abdomen lower quadrants were shaved using an electric trimmer and the area was disinfected with alcohol prep pads. Animals were placed onto Styrofoam pads on their backs, with heads oriented way from the operator. With adequate anesthesia, no restraints were needed.

Surgical procedure

A 1.5 cm longitudinal skin midline incision was made with a scalpel, being careful not to penetrate into the peritoneal cavity. After the initial incision, small scissors were used to extend the incision and to gain entry into the peritoneal cavity. The abdominal linea alba was identified and dissected for intermuscular incision and incision of fascial and peritoneal layers.

The cecum was located by using blunt anatomical forceps to isolate it and exteriorize it, leaving the remainder of the small and large bowel within the peritoneal cavity. It is critical not to breach or damage the mesenterial blood vessels. In the majority of cases, the cecum is found on the left side of the abdomen.[13]

The cecum was ligated at the designated position to induce high-grade sepsis, this procedure shall be done under the ileocecal valve so that intestinal continuity is maintained. Before cecal perforation, the cecal contents were gently pushed toward the distal cecum. The cecum was perforated by single through-and-through puncture with a 21 G needle midway between the ligation and the tip of the cecum in a mesenteric-to-antimesenteric direction.[14,15]

After removing the needle, a small amount (droplet) of feces was extruded from both the mesenteric and antimesenteric penetration holes to ensure patency. The cecum was relocated into the abdominal cavity without spreading feces from the cecum onto the abdominal wall wound margins.

The peritoneum, fasciae and abdominal musculature were closed by applying simple running sutures. The skin was closed by applying simple interrupted sutures.

The animals were resuscitated by injecting prewarmed normal saline (37° C; 5 ml per 100 g body weight) subcutaneously.

The mice were placed back in cages in a temperature-controlled room (22° C) with 12-h light and dark cycles and monitored every 4 h till 120 h completed. Mice were returned to cages immediately at the end of the surgical procedures where access to water and food was available.

SG animals underwent exactly the same procedures except for the CLP procedures, which were omitted in sham animals. Furthermore, the procedure to induce sepsis was carried out at the same time of the day because of circadian rhythm effects on the inflammatory response.[16]

Statistical analysis

Survival rate was assessed by the Kaplan-Meier curve. The survival curves of the different treatment groups were compared by log-rank test. *P* values less than 0.05 were considered to be statistically significant. All statistical analyses were performed with BioEstat 5.0 (IDSM, Belém, PA).

RESULTS

Table I – Survival rates after CLP in mice according to groups.

Day	SG	CG	EG
1st	7 (100%)	5 (71.4%)	6 (85.7%)
2nd	7 (100%)	2 (28.6%)	5 (71.4%)
3rd	7 (100%)	2 (28.6%)	5 (71.4%)
4th	7 (100%)	0 (0%)	5 (71.4%)
5th	7 (100%)	0 (0%)	5 (71.4%)

SG: Sham group; CG: Control group; EG: Experimental group.

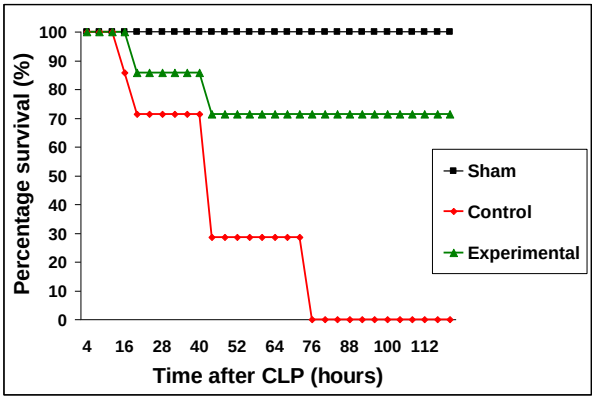

Figure 1 – Survival rates after CLP in mice according to groups (Kaplan-Meier).
$p < 0.001$ (Long-rank test Control versus Experimental)

DISCUSSION

Some in vitro evidences demonstrated that *C. reticulata* Ducke has a microbicidal activity. To evaluate the in vivo anti-microbial effect of this species we used the model of CLP which resembles the clinical situation of bowel perforation and mixed bacterial infection of intestinal origin which seems to be the most realistic model of sepsis.[12]

The model of CLP is widely used and known to closely mimic the pathophysiology of septic human patients. Accordingly, polymicrobial sepsis is associated with an early hyperdynamic phase and a late hypodynamic phase. Similar to septic human patients, rodents with sepsis induced by CLP respond to fluid resuscitation and antibiotics.[17,18]

The onset of sepsis occurs at around 12 h after CLP. The main part of lethality proceeds within the first 48 h, whereas the frequency of death events decreases with time. Lethality began at 16-24 h after CLP, indicating that CLP under the conditions described above represents a rapidly lethal model of acute sepsis. The copaiba oil treatment improved the mice survival when compared to the control group (Figure 1).

All mice were alive 120 h after sham-operation, comproving that the surgical manipulation was carried out at good aseptic conditions.

As previously described, survival rates in high-grade sepsis model are supposed to be 0% in 72 h after CLP,[19] which agrees with findings in this study, CG mice were all dead after third day of sepsis (Table I).

Several mechanisms are involved on survival septic mice, one of them related to the improved survival is the NO decrease, since the exacerbated NO production could result in decrease of neutrophil recruitment.[10] The cholinergic pathway in modulation of overwhelming immune response in sepsis is a new feature to be assessed.[20]

CLP-induced sepsis showed 85.7% survival rate on the first day of observation and reached a stable 71.4% survival rate on the fifth day.

Log-rank analysis of the 5-day survival curves demonstrated that *C. reticulate* Ducke at doses of 0.63 ml per kg provided a significant level of protection and experimental reached a plateau on the second day with a survival rate of 71.4%. Similar results have been found using *Aloe vera*, indicating that it may be a potent and efficacious antiseptic agent.[11]

Our findings suggest that *C. reticulata* Ducke has potent antiseptic effect. Thus, future researches are necessary to investigate protection mechanisms and define properly if it's associated to a direct microbicidal effect or to a recruitment of activated neutrophils to the infectious site and to a diminished systemic inflammatory response.

CONCLUSION

The authors conclude that the septic mice treatment with copaiba oil subcutaneous injection improve survival.

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RESUMO

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Objetivo: analisar a sobrevida de camundongos submetidos à ligadura e perfuração cecal (LPC) tratados com injeção subcutânea de óleo de copaiba. **Método:** pesquisa experimental com vinte e um camundongos machos BALB/c foram distribuídos randomicamente nos três seguintes grupos: Padrão (submetidos à todos os procedimentos cirúrgicos exceto pelos procedimentos de LPC); Controle (submetidos ao procedimento de LPC e sem tratamento); e Experimental (submetidos ao procedimento de LPC e tratados com óleo de copaiba, 0.63 ml por kg, injetado no subcutâneo uma vez ao dia por cinco dias). Sepse foi induzida por LPC e posteriormente os camundongos foram monitorados a cada 6 h até completar 120 h. A sobrevida foi avaliada pela curva de Kaplan-Meier e pelo teste long-rank. **Resultados:** houve diferença significante na sobrevida dos animais que receberam injeção de óleo de copaíba, a taxa de sobrevida dos animais do Controle foi de 0% após 72 h, como esperado, porém a taxa de sobrevida dos animais do Experimental foi de 71.43% até o último período de observação (120h). **Conclusão:** o tratamento com óleo de copaiba aumentou a sobrevida de camundongos submetidos à LPC.

Descritores: Plantas Medicinais; Camundongos; Sepse; Análise de Sobrevida.

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