Experimental empyema in rats through intrapleural injection of bacteria

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Abstract

Objective: to evaluate empyema formation in rats through the injection of two bacteria (Pasteurella multocida and Staphylococcus aureus), using a simple, easy-to-use surgical technique.

Methods: twenty-four anesthetized Wistar white rats, 250-300g in weight, submitted to right anterior thoracotomy, muscular retraction and injection of a 0.2ml solution into pleural space according to the following scheme: Group I (n=12): injection of $10^{10}$ Pasteurella multocida cultured in brain heart infusion broth. Group II (n=8): injection of $10^{10}$ Staphylococcus aureus cultured in brain heart infusion broth. Group III (n=4): injection of bacterium-free brain heart infusion (control). The rats were sacrificed after seven days, and pleural reaction was assessed by macroscopy. Mortality, and intrathoracic liquid volume were evaluated, and bacteriological tests were also performed.

Results: seven rats died within the first 48 hours in Group I (Pasteurella multocida); five completed the experiment, but none of them presented empyema. Only one animal died within the first 24 hours in Group II (Staphylococcus aureus); seven (88%) presented empyema at the time of sacrifice. All animals survived in Group III (control), without empyema or thoracic abnormalities. Pleural inoculation of Staphylococcus aureus (Group II) was significantly associated with empyema formation ($P<0.001$). In this group, the amount of pleural liquid ranged from 0.9 to 3.9ml.

Conclusion: it is possible to induce empyema in rats through Staphylococcus aureus pleural injection by a simple surgical technique. Differently from other experiments, the pleural injection of Pasteurella multocida did not provoke empyema in rats.


Introduction

Empyema, which is described as the accumulation of pus in the pleural space, is responsible for high morbidity and mortality rates in both children and adults. The treatment of empyema varies according to the protocols of different medical centers, especially due to the lack of clinical assays that are appropriately carried out in humans. This is a result
Sasse et al.\textsuperscript{1} induced empyema by injection of the bacterium Pasteurella multocida in brain heart infusion (BHI) agar into the right hemithorax of New Zealand white male rabbits. Despite being the model that more closely mimics the empyema that affects humans,\textsuperscript{3-6} there are some difficulties to apply this experimental model in our settings. Tonietto et al.\textsuperscript{7} published a recent study on induced empyema in rats by intrapleural inoculation of various concentrations and species (Staphylococcus aureus, Escherichia coli and Bacteroides fragilis). The guinea pigs were divided into three groups of 30 pigs. Results of the study indicated successful induction of empyema in 58\% of animals in the Staphylococcus aureus group and in 37\% of those in the Escherichia coli group; there were no cases of induced empyema in the Bacteroides fragilis group. The objective of the study was not exclusively of describing empyema, but also of testing the effect of concomitant hemothorax.\textsuperscript{2}

Our objective was to assess the induction of experimental empyema in rats by intrapleural inoculation of two bacteria (Pasteurella multocida and Staphylococcus aureus) using a simple and easy surgical technique.

**Methods**

We carried out a controlled experimental study at the Desidério Finamor Veterinary Research Center using isolated animal facilities specific for contamination studies. We selected 24 adult Wistar albino rats of both sexes; animals weighed between 250 and 300 g and were obtained randomly from the same breeder. Animals were divided into three main groups according to the colony of bacteria used in the inoculation:

- **group 1** (n=12): inoculation into the pleural space of 0.2 ml of Pasteurella multocida solution at \(10^{10}\) colony-forming units (CFU) per ml diluted in BHI; without learning about the behavior of the animal following exposure to bacteria (especially early loss of the model due to infection), the rats were divided into two subgroups according to administration of antibiotic therapy, which started following the postoperative recovery period;
  - subgroup 1a (n=8): amoxicillin at 0.05 or 0.1\% concentration diluted in liquid, administered orally and ad libitum;
  - subgroup 1b (n=4): no antibiotic therapy in liquids administered orally and ad libitum;
- **group 2** (n=8): inoculation into the pleural space of 0.2 ml of Staphylococcus aureus solution at \(10^{10}\) colony-forming units (CFU) per ml diluted in BHI;
- **group 3** (n=4): inoculation into the pleural space of 0.2 ml of sterile BHI.

The Pasteurella multocida bacteria were obtained from the laboratory of the Desidério Finamor Veterinary Research Center. The Staphylococcus aureus bacteria were collected with swabs and isolated from the oral mucosa of animals; cultures of these bacteria were made at the same laboratory.

The procedures of the experiment were based on two different stages of assessment. Firstly, we tested the experimental model of induction of empyema in rats using only Pasteurella multocida inoculation (n=12) and the respective controls (n=2). Secondly, we used Staphylococcus aureus inoculation (n=8) and the respective controls (n=2).

Our study was approved by the Research and Graduate Studies Group of the Hospital de Clínicas de Porto Alegre and by the Desidério Finamor Veterinary Research Center. Throughout the study, the rats were treated according to the specific protocols for handling and care of laboratory animals.

**Induction of empyema**

Animals were placed in sealed glass chambers and anesthetized by inhalation using pledgets damped with ethyl ether. Anesthesia was maintained using intraperitoneal sodium pentobarbital at 25 mg/kg. Rats were positioned supine and submitted to trichotomy to the anterior thoracic region. Antiseptic procedures included use of alcohol iodophor and use of sterilized material. Thoracotomy was carried out with right oblique incision to the hemithorax and with separation of musculature and exposure of intercostal spaces. We opened the fourth intercostal space using hemostatic forceps and then inoculated the rats with the bacteria. After this stage was concluded, we released the musculature previously separated by the forceps and sutured the rats using mononylon 4-0. All procedures were carried out observing asepsis techniques.
Assessment of empyema

All animals were sacrificed by intraperitoneal administration of a lethal dose of sodium pentobarbital. Animals that died before the conclusion of the experiment were submitted to necropsy for presence of empyema; we also collected liver and spleen tissue samples for culture.

The empyemas were characterized according to pleural fluid presenting as a turbid exudate and identification of microorganism following bacteriologic examination of the material.

Macroscopic analysis of pleural alterations

Animals were sacrificed three, five, or seven days following pleural inoculation.

Animals in group 1 were sacrificed in seven days. Half of the animals in group 2 were sacrificed in three days and the other half in five days. In the control group (group 3), half of the animals were sacrificed in five days and the other half in seven days.

After the animals were sacrificed, we analyzed pleural abnormalities macroscopically according to a score of severity of pleural reaction (Table 1).

Table 1 - Macroscopic grading of pleural reaction*

<table>
<thead>
<tr>
<th>Grade</th>
<th>Macroscopic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No adherence or pleural fluid</td>
</tr>
<tr>
<td>1</td>
<td>Turbid fluid, with adherence at the site of surgical incision</td>
</tr>
<tr>
<td>2</td>
<td>Purulent fluid containing fibrin, with moderate pleural adherence</td>
</tr>
<tr>
<td>3</td>
<td>Dense and well-developed adherence, with or without pleural fluid</td>
</tr>
<tr>
<td>4</td>
<td>Encapsulated empyema with fibrosis</td>
</tr>
</tbody>
</table>

* Adapted from Tonietto et al.7

Statistical analysis

The variables that characterize the sample were described in absolute and percentage values; we also calculated the averages and standard deviations. The differences in average fluid volume were compared using Student’s t test for nonpaired samples. We tested association between inoculated bacteria and induction of empyema using the chi-squared test. We considered a significance level of $P = 0.05$.

Results

Figure 1 presents the distribution of animals in all three groups according to survival after intrapleural inoculation of the bacteria and conclusion of the experiment (sacrifice of animals).

In group 1 (n=12), seven rats died in the first 48 hours of the experiment. Five rats were sacrificed within the predetermined period of one week, but without presenting any evidence of empyema. One of the rats presented fibrosis of the chest wall and the other four did not present any abnormalities following macroscopic examination of the chest wall. Individual analysis of the subgroups in the experiment indicated that use of antibiotics in subgroup 1a neither decreased mortality nor contributed to induction of empyema (Figure 2).

In group 2 (n=8), only one rat died in the first 24 hours after inoculation of the bacterial agent (*Staphylococcus aureus*). All other rats (n=7; 88%) survived the experiment and presented evidence of empyema by macroscopy. There were no differences in findings according to day of sacrifice; however, the average fluid volume collected tended to be greater in animals examined three days after bacterial inoculation (3.5 ± 0.1 ml x 2.0 ± 2.8 ml; medians = 3.5 and 1.1, respectively). This difference was not statistically significant (P=0.09). The total fluid volume collected ranged from 0.9 ml to 3.9 ml. The fluid culture presented growth of *Staphylococcus aureus*.
Grading macroscopic findings

<table>
<thead>
<tr>
<th>Group</th>
<th>(n=12)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td>12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Group II</td>
<td>(n=8)</td>
<td>1</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Group III</td>
<td>(n=4)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

P < 0.001

Discussion

The definition of an appropriate treatment for empyema is controversial; it is usually based on personal experience and on a limited number of cases reported in the literature. Choice of surgical procedures can be influenced by several variables, such as age of patient, clinical status, response to antibiotic therapy, microorganisms detected in culture, stage and duration of empyema on presentation. The spectrum of possible treatments includes: antibiotics alone or associated with thoracentesis; chest drainage procedure (chest tube placement or open drainage procedure); fibrinolitics; thoracoscopy; minithoracotomy; thoracotomy; and decortication (1,8,9). In this sense, experimental models of empyema are needed to carry out prospective studies in which the several treatment options are evaluated. These models can also allow for studies on specific stages of empyema, for the control of confounding clinical variables, and for the establishing of an adequate population of animals that will yield more statistical power to the analyses.

Figure 2 - Survival rate and sacrifice of animals after inoculation (Time 0) of Pasteurella multocida (Group I), considering the use or nonuse of antibiotics

In group 3 (n=8), or control group, all animals survived; we did not find evidence of pleural fluid or thoracic abnormalities when the animals were sacrificed.

Tissue culture (spleen and liver) results from rats that died before the sacrifice indicated presence of the same bacteria that was previously inoculated into the intrapleural space. Table 3 presents the distribution of macroscopic findings on pleural reactions according to the severity score.

Table 2 - Macroscopic findings at the time of death or at the time of sacrifice of animals (according to grades presented in Table 1)

Light has studied empyema experimentally more extensively than any other author.1,3,8 The author developed an experimental model with rabbits using intrapleural administration of Pasteurella multocida. The inoculation is carried out by positioning a catheter into the pleural space.3,6,8 Due to the extreme vulnerability of rabbits (early death due to sepsis) parenteral antibiotics (penicillin) were administered daily. There is an operational inconvenience related to carrying out this procedure in our settings: the cost of the animal is elevated and they demand specific care that, usually, requires more sophisticated laboratory facilities to keep the rabbits free from diseases. Other centers have successfully reproduced this model during specific investigations on the management of empyema.6 Though minimally invasive and of easy technical application (catheterization and inoculation), the experimental model requires monitoring of a characteristic pleural pressure tracing (transducer connected to the catheter and transducer signal displayed on an oscilloscope) to verify that the catheter is properly positioned in the pleural space.3,6

We designed this study in order to try to develop an experimental model with rats that is more agreeable to our reality, considering that these animals represent a low cost for the study, are resistant, and easy to maintain in our environment.

Recently, Tonietto et al.7 induced empyema in rats by instillation of Staphylococcus aureus into the pleural space. The authors isolated the bacteria from animal saliva cultured in a laboratory. Animals were submitted to general anesthesia, tracheal intubation, and thoracotomy for exposure of the pleural region. Despite the fact that Tonietto et al. applied an animal model more suitable to our reality, the surgical technique applied, which required tracheal intubation and mechanical ventilation of rats, rendered the model sophisticated and, consequently, difficult to reproduce.

Our experimental model applies a simpler technique. We used rats as the animal model, but the anesthetic technique does not require tracheal intubation or use of mechanical ventilation. The surgical approach is similar to that reported previously using thoracotomy;7 however, it did not require submitting the rats to tracheostomy to obtain airway access nor did it require mechanical ventilation. The technical procedure applied in our experiment was simple.
and safe. Considering that all controls survived the surgical procedure (group 3), there were no cases of death caused by the small residual pneumothorax present in some of the cases after thoracotomy. This finding is important, considering that, differently from other experimental models, we did not have to worry about approximation of the thoracic musculature or aspiration of residual air within the pleural space before suturing the skin.

Initially, we used *Pasteurella multocida* considering that it is harmless to humans and that it is the most widely used bacteria in induced empyema on other animal models; hence, we had no previous information on infectivity of the bacteria on rat models. The significant mortality of animals before 48 h following inoculation, and the liver and spleen cultures indicating presence of the bacteria, suggests that the rats died due to sepsis caused by the inoculated bacteria. We observed a significant mortality following the inoculation, independently of administration of antibiotics. The formation of a subgroup with daily use of oral antibiotics also resulted in some difficulties. Despite the erratic absorption of oral antibiotics, the alternative of daily administration of parenteral antibiotics, as proposed by Sasse et al., would have required daily anesthesia of the animal, thus rendering the experimental model complex and defeating the purpose of this study. Consequently, we opted for administration of oral antibiotics. Even with high doses of antibiotics, we were not able to induce empyema after inoculation of *Pasteurella multocida* into the pleural space of rats, since the animals continued to die early of septicemia. It is possible that the oral administration of antibiotics was not ideal in terms of absorption and, also, that the animals died considering that therapeutic levels of the drugs were not reached; however, not even the animals that survived developed empyema.

During the second stage of our experiment, the animals were inoculated with *Staphylococcus aureus* and we observed the presence of turbid pleural fluid in the sacrificed animals on both the third and fifth days after the inoculation. Culture from the collected fluid indicated presence of *Staphylococcus aureus* and thus empyema.

Since we are dealing with an experimental study, there are methodological considerations that need to be made. One of the potential limitations of the study is related to the comparison of the findings from groups 1 and 2, especially considering that the analysis of the macroscopic findings was carried out with animals sacrificed on different days. Despite this potential limitation of the study, we were not able to show findings compatible with empyema in any of the animals in group 1. This finding indicates better than any comparison that inoculation of the *Pasteurella multocida* bacteria into the pleural space of rats is not an adequate experimental model of empyema, considering the techniques applied in this study. Our main objective was to design a new experimental model for such a prevalent respiratory disease as the empyema; we were successful in developing an animal model with the inoculation of *Staphylococcus aureus* that required a much simpler technique than those reported by others.

The choice of sacrificing animals at an earlier time in group 2 (*Staphylococcus aureus*) was made based on the possibility of identifying more subtle pleural manifestations that may occur at an earlier time in this animal model, considering the results obtained during the first stage of the experiment. We understand that there were no such findings, since the macroscopic findings and the appearance and volume of the fluid collected suggest a significant inflammatory pleural response on both the third and fifth day after inoculation.

The surgical technique applied (thoracotomy) and the dilution of bacteria in BHI before inoculation did not result in any complications. This can be verified in the control animals (group 3) that survived until the end of the experiment and did not present abnormalities on macroscopic examination at necropsy. In a sense, this decreases the possibility of biased results considering that we did not carry out a randomized experiment; in other words, the possibility that we influenced an increased mortality and/or failure in the procedures carried out with the first group.

Our study showed that it is possible to carry out thoracotomy and pleural inoculation of bacteria in anesthetized rats using a simple technique, which requires neither tracheal intubation nor mechanical ventilation. The fact that the model does not require mechanical ventilation allows for a good operational feasibility, even in animal research centers with less technological resources. In this sense, also, the fact that it does not require tracheostomy to obtain access to the airways decreases the complexity of, and time consumed with the intervention, which might allow for decreased morbidity. In our animal model, the inoculation of $10^{10}$ CFU of *Pasteurella multocida* into the pleural space was extremely virulent, leading to early death of most animals. Also, we were not able to observe induced empyema in the rats that survived the *Pasteurella multocida* inoculation, thus indicating that this is not an appropriate model according to the objectives established. However, using the same animal model with inoculation of $10^{10}$ CFU of *Staphylococcus aureus* successfully induced empyema, which was verified on both the third and fifth days after the surgical procedure. These findings allow for new perspectives for experimental studies aimed at a better assessment and handling of empyema.

References


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