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Experimental pleural empyema model in rabbits: Why, how and what are the next steps

Eksperimentalni model empijema pleure kod kunića: zašto, kako i šta dalje

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Abstract

Bacgraund/Aim. The use of new therapeutic methods to prevent development of fibrothorax as the final complication of the human pleural infections requires research with experimental animals. The aim of this study was to standardize the procedures for the establishment of our own experimental model of empyema in rabbits, since it should be able to offer similar conditions found in human pleural infections. **Methods.** This experiment included 15 chinchilla rabbits, weighing from 2.3 to 2.8 kg. There were 12 rabbits in the experimental group, while 3 rabbits formed the control group. On the first day, we administered 0.4–0.5 mL of turpentine in the right pleural space of the rabbits from the experimental group in order to provoke sterile exudative pleurisy. After 24 h we injected 1 mL of *Staphylococcus aureus* and 1 mL of *Escherichia coli* bacteria in the same concentration of 4.5×10^8 bacteria/mL. Thoracocentesis for the pleural fluid analysis was performed 24, 48, 72, and 96 h after bacteria instillation. In these pleural samples we estimated the number of leucocytes and the values of lactate dehydrogenase (LDH), glucose and pH in pleural fluid, as well as the presence of bacteria. We did not protect the animals with antibiotics, and on the day 7 of the experiment they were sacrificed with the lethal dose of barbiturate (*iv*). The lung from the empyemic side of all experimental animals and the lung of one control animal were histopathologically examined. **Results.** A total of 4 animals had a small amount of clear pleural fluids or there was no fluid obtained with thoracocentesis 24 and 48 h after the bacteria instillation. after the bacteria instillation. In the remaining 8 rabbits 24 h after bacteria administration the mean values (± SD) of the parameters monitored were as follows: Le $34.75 \pm 6.13 \times 10^9$/L, LDH 17,000 ± 4,69 U/L, glucose 1.23 ± 0.45 mmol/L, and pH 6.975 ± 0.15. The obtained values met the criteria for the evaluation of effusion as pleural empyema or complex and complicated pleural effusion (LDH > 1000 U/L, glucose < 2.31 mmol/L and pH < 7.20). Bacterial cultures were positive in 5 out of 8 first pleural samples and in only 2 samples after 48 h of bacteria administration. There was a positive correlation between the number of leukocytes and the LDH value ($r = 0.071$, $p < 0.001$), and a negative correlation between the number of leukocytes and the glucose level ($r = 0.864$, $p < 0.001$), and the leukocytes number and pH of the pleural fluid ($r = 0.894$, $p < 0.001$). The mean glucose value increased after 48 h (3.23 ± 0.44 mmol/L), and the pH value rose after 72 h (7.22 ± 0.03) which was beyond the empyema level. **Conclusion.** The creation of the experimental empyema model is a very delicate work with uncertain success. Its value and importance are crucial for pleural pathology research. With the intention to obtain a more empyemic pleural reaction we created a model with two different human pathogen bacteria. We generated the satisfactory results, but not as good as those contained in some of the reference literature data.

Key words:
empyema, pleural; animal experimentation; rabbits; pleural effusion; bacteria.

Apstrakt

Uvod/Cilj. Primena novih metoda lečenja u cilju sprečavanja razvoja fibrotoraksa, kao krajnje komplikacije empijema zahteva prethodno ispitivanje na eksperimentalnim životinjama. Cilj rada bio je standardizovanje postupaka za ustanovljavanje pouzdanog eksperimentalnog modela empijema kod kunića. **Metode.** U eksperimentu je korišćeno 15 činčila kunića mase od 2 300 do 2 800 g. Eksperimentalnu grupu činilo je 12 kunića, a kontrolnu tri. Prvog dana eksperimenta, u desni pleuralni prostor kunića iz eksperimentalne grupe stavljeno je 0,4–0,5 mL terpentina u cilju izazivanja sterilnog eksudativnog pleuritisa. Nakon 24 sata u pleuralni prostor stavljen je 1 mL *Staphylococcus aureus-a* i 1 mL *Escherichia coli* bakterija iste koncentracije ($4,5 \times 10^8$ bakterija/mL). Torakocenteza je rađena 24, 48, 72 i 96 časova nakon primene bakterija radi dobijanja

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uzoraka pleuralne tečnosti. U ovim uzorcima određivane su vrednosti leukocita (Le), laktat dehidrogenaze (LDH), glukoze, pH u pluralnoj tečnosti i prisustvo bakterija. U toku eksperimenta životinje nisu dobijale antibiotik, a žrtvovane su sedam dana od primene bakterija letalnom dozom barbiturata *iv*. Patohistološki su pregledana sva pluća sa empijemom svih eksperimentalnih životinja, kao i pluća jedne kontrolne životinje. **Rezultati.** Kod četiri životinje dobijen je oskudan bistar sadržaj, ili sadržaja nije bilo prilikom punkcija 24 i 48 časova od primene bakterija. Kod preostalih 8 eksperimentalnih životinja srednja vrednost Le u izlivu 24 časa nakon primene bakterija bila je: $34,75 \pm 6,13 \times 10^9$/L, LDH $17,000 \pm 4,69$ U/L, glukoze $1,23 \pm 0,45$ mmol/L, a pH $6,975 \pm 0,15$. Navedene vrednosti ispunjavaju kriterijume za proglašavanje izliva empijemskim ili kompleksnim, komplikovanim izlivom (LDH > 1 000 U/L, glukoza < 2,31 mmol/L i pH < 7,20). Bakteriološke kulture bile su pozitivne kod pet od osam prvih uzoraka, a nakon 48 časova samo kod dva uzorka pleuralnog izliva. Utvrđeno je postojanje pozitivne korelacije između broja leukocita i vrednosti LDH ($r = 0,071$, $p < 0.001$), a negativne korelacije između broja leukocita i vrednosti glukoze ($r = 0,864$, $p < 0.001$) i broja leukocita i vrednosti pH izliva ($r = 0,894$, $p < 0.001$). Vrednost glukoze nakon 48 sati ($3,23 \pm 0,44$ mmol/L) i pH nakon 72 sata ($7,22 \pm 0,03$), izašle su iz okvira empijemskih vrednsoti. **Zaključak.** Kreiranje eksperimentalnog modela empijema veoma je delikatan posao, sa neizvesnim uspehom. Dobar eksperimentalni model od suštinskog je značaja za proučavanje pleuralne patologije. U cilju što boljeg pleuralnog odgovora napravili smo model sa dve humane patogene bakterije. Dobili smo zadovoljavajuće rezultate, mada slabije od onih koji su objavljeni u literaturi.

Ključne reči:
empijem; životinje, zaštita; zečevi; pleura, izliv; bakterija.

Introduction

Pleural empyema is a serious inflammatory disease which is characterized by the presence of purulent effusion in the pleural space and, almost always arises as a complication of some other disease or condition. The clinic phases of empyema are acute, intermediate and chronic and these are counterparts of exudative, fibropurulent and fibrous pathologic stage of the disease [1, 2].

Despite effective antibiotic therapy and different drainage options which are available today for drainage of pleural infectious space, pleural empyema remains a serious medical problem with morbidity and mortality up to 20%. Half of all empyemas are consequences of inappropriate pneumonia treatment. Other causes may be a thoracic trauma, surgical procedures and infection spreading from the surrounding organs [3–5].

Pleural effusion is found in 9–30% of cases of bacterial pneumonia. The frequency is much higher among hospitalized patients (33–66%). Patients with unilateral and bilateral parapneumonic effusion have 3.7 and 6.5 times higher mortality rate, respectively [6, 7].

Thoracic drainage along with antibiotics is the most frequent initial empyema treatment. The choice of treatment modality depends on the severity of the disease and the stage of illness at the moment of diagnosis, as well as on patient's overall condition.

In addition to the thoracic drainage there are other treatment modalities, such as: drainage with intrapleural application of fibrinolytics, video-assisted thoracoscopic surgery (VATS) debridement, thoracotomy with decortication, open drainage and thoracoplasty [8–10]. The main goal of empyema treatment is cleaning of purulent pleural content, followed by lung reexpansion intending to avoid complications and the need for second operation [1, 2, 11].

Despite the fact that up to 20% of pleural parapneumonic effusions progress to empyema and that up to 40% of empyemas need a more aggressive surgical treatment, most of thoracic surgery centers usually have a few empyema patients. That is why there are very few randomized prospective trials with the purpose to compare efficacy of empyema treatment modalities [12].

The first experimental empyema model was made by Graham and Bell and they performed it on dogs almost a century ago. The rabbit turpentine model, the pig model with umbilical cord and the rabbit model with nutritive agar were made in the last 30 years of the last century. These models were used for the basic empyema research at the beginning of the last century [13], for studying therapeutic and drainage procedures efficacy [3, 14] and antibiotics used in the empyema treatment [9, 15].

Richard W. Light established well-known criteria for parapneumonic effusions and empyemas classification. A parapneumic effusion will be classified as an empyema if its glucose level is lower than 40 mg/dL or 3.0 mmol/L, pH value under 7.0 and lactate delydrogenase (LDH) value above 1000 I/U. Beside these biochemical parameters, positive bacteriological cultures or frank pus in pleural space have the same importance [16].

In the last few years the experimental empyema model has been used for basic research again, but this time on the cellular level, studying the role of some pro-inflammatory and pro-fibrotic cytokines and possibilities of the therapeutic use of their antagonist or inhibitors.

Most promising is the use of transforming growth factor-β (TGF-β) antagonists to prevent proliferation of fibroblasts and deposition of extracellular matrix which are responsible for fibrothorax formation [16].

It is obvious that therapeutic potentials of the abovementioned surgical procedures leave room for the research of new substances, which could be applied in empyema treatment. Our goal was to make a reliable experimental empyema model that would enable us to participate and continue these studies.

Methods

Experimental animals

The research was approved by the Military Medical Academy Ethics Committee. We used 15 four-month-old male chinchilla rabbits, weighing 2.3-2.8 kg. The rabbits

were from the Institute for Medical Research farm of the Military Medical Academy. They were placed in separate boxes. They were given food and water *ad libitum*. We randomised two groups: the experimental one with 12 rabbits with intrapleural application of bacteria, and the control one with 3 rabbits without application of bacteria.

Bacteria preparation

In this experiment we used *Staphyloccocus aureus* ATCC 25923 strain, and *Esherichia coli* ATCC 25922 strain. Concentration of the bacteria was determined under McFarland scale (Biomerieux, Densi chek plus McF). We administered 1 mL saline solution with 4.5×10^8/mL (1.5 McF) *Staphyloccocus aureus* and 1 mL saline solution with *Esherichia coli* bacteria in the same concentration. The day before the application bacteria were seeded on fresh blood agar.

Empyema induction

The rabbits from the experimental group were anesthetized with ketamine, 35 mg/kg, (Laboratorio Sanderson, Santiago Chile), and acepromazine 0.1 mg/kg (Ceva Tiergesundheit GmbH Dusseldorf, Germany). The right chest wall from the sternal to scapular line of each rabbit was shaved and scrubbed with povidon iodine (Betadine®).

The animals were placed in the supine position on the operating table. With the surgical knife, we made a small, 5 mm long incision, between the middle and lower third of the right hemithorax, 2.5–3 cm from the sternal edge. We performed thoracocentesis with a Mediflon *iv* catheter 18 G × 1.3 × 45 mm, flow 90 mL/min (Eastern Medikit Ltd. Gurgaon India). After we had excluded the presence of pneumothorax with probe aspiration, we injected 0.5–0.6 mL of turpentine through the catheter in the right pleural space of each experimental rabbit. After turpentine application the catheter was rinsed with 0.5 mL of saline solution. The aim of turpentine application was to cause a chemical pleural lesion followed by sterile effusion. Skin suture was not necessary. We did not inject turpentine and bacteria into the rabbits from the control group and did not perform any thoracocentesis.

After 24 h we conducted the same anesthesia procedures and thoracocentesis and then 8 experimental rabbits were administered 2 mL of saline solution with the abovementioned bacteria concentration. The catheter was rinsed with 0.5 mL of saline solution. The animals were gently rotated manually in order to spread the bacterial solution uniformly in the pleural space.

Experimental animals were given 50 mL of saline solution and 50 mL of 5% glucose solution deep in the subcutaneous tissue on a daily basis in order to prevent dehydration.

Empyema verification and pleural specimens

After 24 h applying bacteria 8 rabbits from the experimental group were anesthetized again, and subjected to thoracocentesis. We evacuated 2 mL of pleural effusion from each rabbit and divided this specimen into 4 parts: for microbiological, biochemical, immunological and hematological analysis. We analyzed values of with blood cells (WBC), LDH, glucose and pH of the effusion. Pleural fluid samples were put into blood agar in order to observe bacterial colonies growth. Empyema was considered where the effusion specimen was apparently purulent, or if the glucose was < 2.3 mmol/L (normal range: 4.1–5.9 mmol/L), pH < 7.10 and LDH > 1 000 U/L. Both lungs of all experimental animals were histopathologically examined.

Glucose and LDH values were measured under the Advia 1800 Chemistry System, (Siemens Germany), and WBC under the Advia Hematology System (Siemens Germany) device. Pleural fluid pH values were measured on a digital pH meter (Model 4500, Beckman, USA). Thoracocentesis in 8 experimental rabbits was performed 24, 48, 72 and 96 h after the bacteria administration.

Animals' well-being and the end of experiment

The experiment lasted 7 days and in this period we respected all the principles of animals' well-being. All experimental animals were sacrificed with a lethal intravenous dose of pentobarbital injected through the ear vein, then we performed autopsy and took lungs for histopathological examination.

Histopathological examination

The lungs from empyemic side of all experimental animals and the lung of one control animal were taken for histopathological examination.

We used 4 tissue samples of each rabbit lung specimen, parafinized them and hematoxylin eosin (HE) stained. We randomized 5 HE slides of each tissue sample and did microscopic measurement on 4 microscopic fields. In that way we had done 20 measurements of pleural thickness of each tissue sample.

Statistical analysis

The results were presented as mean values with standard deviation. The significant differences between more than 2 groups were analyzed using one-way ANOVA test (*post-hoc* Tukey test). The relationships among variables were evaluated by Pearson's correlation analysis. A p value of less than 0.05 was taken to be significant. The obtained data were processed through the Star for Windows, R.4.5. software package.

Results

In 4 rabbits the pleural specimens were scarce and clear or there was no pleural effusion following the thoracocentesis 24 and 48 h after the bacteria administration. After the autopsy we did not find any intrapleural changes other than pleural congestion with a few mL of clear effusion and intense smell of turpentine. These animals were excluded, and there were 8 rabbits left in the experimental group.

The mean values ± SD of leukocyte count, LDH, glucose and pH in the pleural fluid specimens obtained by thoracocentesis in 8 rabbits 24, 48, 72 and 96 h after the bacteria administration are shown in Table 1. The dynamics of leukocyte, glucose and pH values alterations in the function of time are shown in Figures 1, 2 and 3.

Table 1

Values of pleural fluid parameters for 8 rabbits over time induced by *Staphylococcus aureus and Escherichia* coli application

Parameters	Hours			
	24	48	72	96
WBC ($\times 10^9$/L), $\bar{x} \pm SD$	34.75 ± 6.13	27.62 ± 4.62	15.62 ± 4.20	5.62 ± 2.87
LDH (U/L), $\bar{x} \pm SD$	17.00 ± 4.69	10.12 ± 4.51	6.50 ± 2.72	4.00 ± 1.92
Glucose (mmol/L), $\bar{x} \pm SD$	1.23 ± 0.45	3.23 ± 0.44	4.15 ± 0.42	4.83 ± 0.46
pH, $\bar{x} \pm SD$	6.97 ± 0.15	7.10 ± 0.09	7.22 ± 0.03	7.28 ± 0.02

WBC – white blood cells; LDH – lactate dehydrogenase.

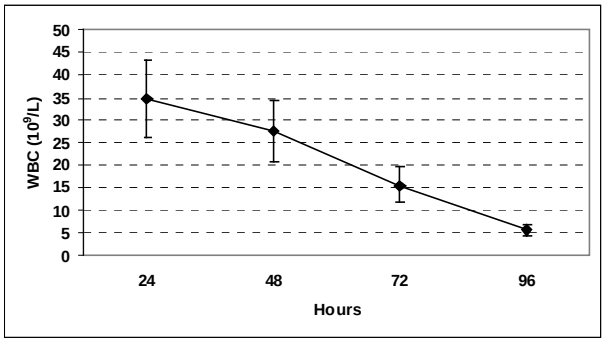

Fig. 1 – The values ($\bar{x} \pm SD$) of pleural fluid leukocytes count *vs* time after intrapleural bacterial injection in 8 rabbits. WBC – white blood cells.

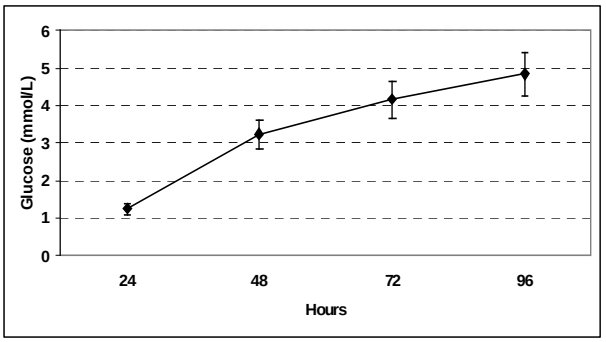

Fig. 2 – The values ($\bar{x} \pm SD$) of pleural glucose *vs* time after intrapleural bacterial injection in 8 rabbits.

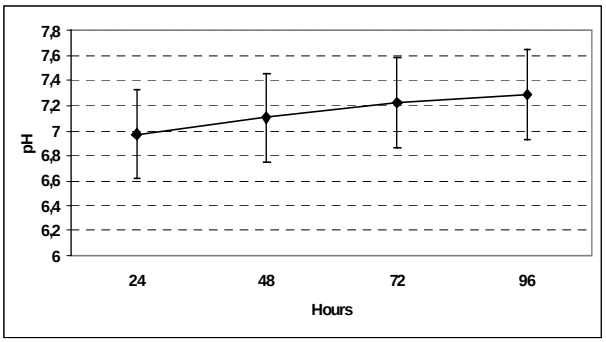

Fig. 3 – The values ($\bar{x} \pm SD$) of pleural effusion pH *vs* time after intrapleural bacterial injection in 8 rabbits.

The mean value of leukocytes found after 24 h was: 34.75 ± 6.13 $\times 10^9$/L, LDH 17,000 ± 4.69 U/L, glucose 1.23 ± 0.45 mmol/L and pH 6.97 ± 0,15. These values entirely met the Light's criteria for complex and complicated parapneumonic effusions and empyema, and 5 of 8 samples showed bacterial growth on blood agar.

The mean value of leukocytes found in pleural fluid after 48 h was: 27.62 ± 4.62 $\times 10^9$/L, LDH 10.12 ± 4.51 U/L, glucose 3.23 ± 0.44 mmol/L and pH 7.10 ± 0.09. High levels of leukocytes and LDH were retained, and low values of glucose and pH were the consequence of intense glucose metabolism and a higher production of acid metabolic products (Figures 2 and 3). Two of eight specimens showed bacterial growth on the blood agar plate.

After 72 and 96 h the mean glucose and pH values were 4.15 ± 0.42 mmol/L, 4.83 ± 0.46 mmol/L and 7.22 ± 0.03, 7.28 ± 0.02, respectively, coming out from the empyema limits. Leukocytes dropped to 15.62 ± 4.20 $\times 10^9$/L after 72 h, and came into the normal range of 5.62 ± 2.87 $\times 10^9$/L 96 h after the bacteria injection. There was no bacteria growth after 72 and 96 h.

We found a significant statistical difference for all the values of leukocytes as well as glucose at any thoracocentesis time ($p < 0.05$). No significant statistical difference was found between LDH values measured after 48 and 72 h as well as 72 and 96 h after the bacteria injection. There was no significant statistical difference for pH values in the pleural samples after 24–48, 48–72 and 72–96 h from bacteria administration.

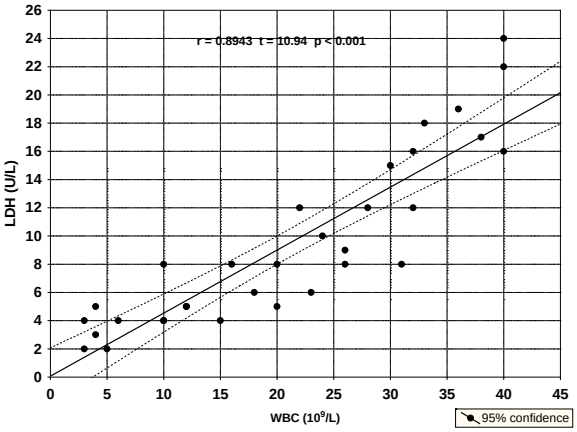

Fig. 4 – A positive correlation between leukocytes count and lactate dehydrogenese (LDH) value in experimentally induced pleural effusion in 8 rabbits.

We found a positive correlation between leukocytes count and the LDH values ($r = 0.071$, $p < 0.001$) (Figure 4), but a negative correlation between leukocytes count and the glucose level ($r = 0.864$, $p < 0.001$) (Figure 5) and between leukocytes count and the pH level in the pleural effusion ($r = 0.894$, $p < 0.001$) (Figure 6).

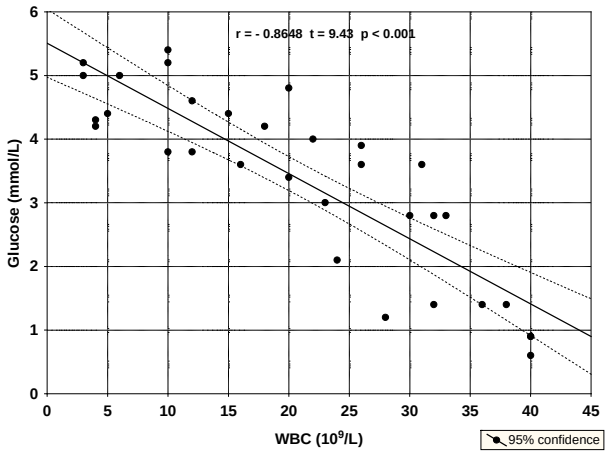

Fig. 5 – A negative corelation between glucose level and leukocytes count in experimentally induced pleural effusion in 8 rabbits.

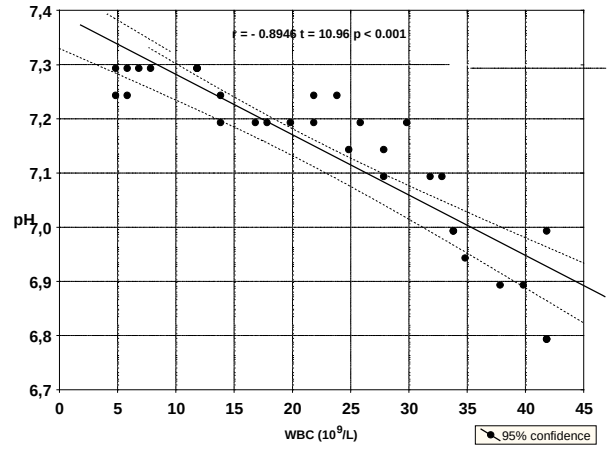

Fig. 6 – A negative correlation between leucocytes count and pH value in experimentally induced pleural effusion in 8 rabbits.

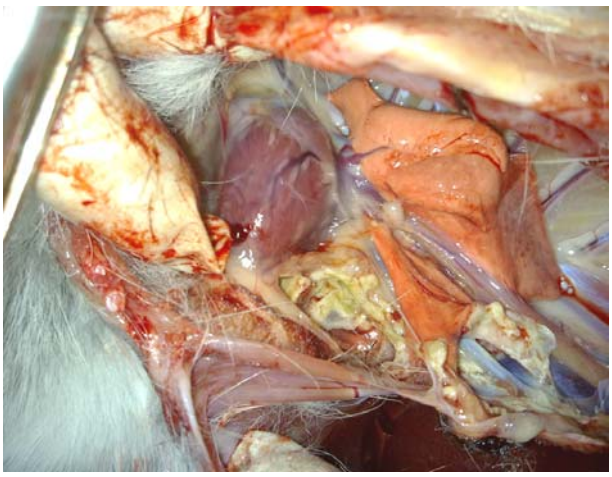

Fig. 8 – Empyemic space with fibrin and purulent detritus. The control lung did not sustain any macroscopic change.

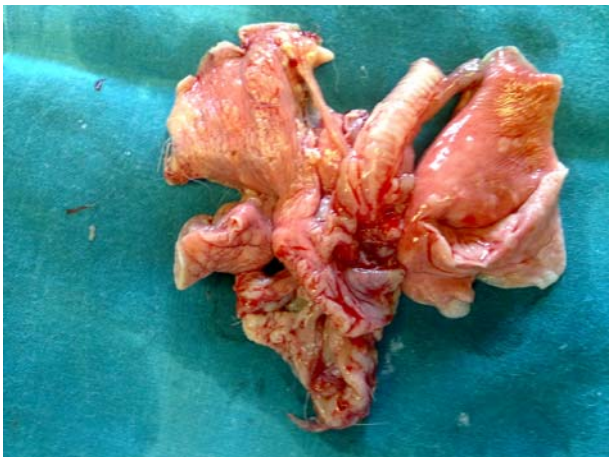

Fig. 9 – Experimentally induced purulent pleural infection causes inflammatory infiltration in subvisceral part of the lung parenchima at the point of the thickest fibropurulent layer.

The animals from the experimental group and one rabbit from the control group were sacrificed on the day 7 of the autopsy. Five of 8 rabbits had purulent effusion, detritus and fibrin layers in the pleural space (Figures 7 and 8). In 3 animals pleural adhesions were dominant with a few millilitres of turbid effusion (Figure 9).

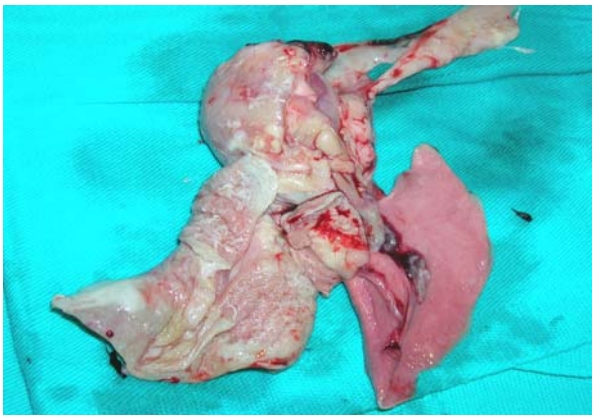

Fig. 7 – Rabbit empyema lung covered with fibrin layers and purulent detritus.

Microscopic examination of HE slides revealed thickness of visceral pleura with prevalent granulocytes infiltrate, fibroblasts and endotel cells proliferation along with purulent detritus on the pleural surface (Figure 10).

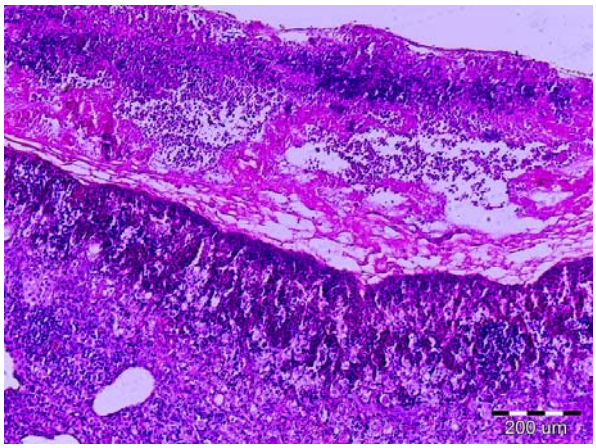

Fig. 10 – Thickened visceral pleura because of submesothelial inflammatory infiltration (HE stain, ×5).

The mean value ± SD for visceral pleura thickness in the experimental group (n = 8 rabbits) was 144.18 ± 24.64 μm. The mean value ± SD of fibroblast number in thickened visceral pleura of the experimental group (n = 8 rabbits) was 196.27 ± 41.20. Macroscopic intrapleural and microscopic lung findings of the control animal were quite normal and the mean value of 20 measurements for visceral pleura thickness was 38.54 μm and the mean value for fibroblast number was 11.30.

Discussion

Despite the extensive experience in the use of Büllau underwater thoracic drainage and decortication, as well as the introduction of new treatment methods such as fibrinolytic therapy video-assisted thoracoscopic surgery (VATS), empyema patients still remain a serious medical problem and therapeutic challenge [17–19].

The American College of Chest Physicians published a clinical practice guideline on the medical and surgical treatment of parapneumonic effusions (PPE), and after comprehensive analysis they found only 3 relevant randomized controlled studies [20]. Ten years after this meta analysis, the situation in this field has not changed substantially [11, 3].

It is well-known that 5–20% of hospitalized patients with parapneumonic effusions progress to empyema, and no thoracic surgery center has enough patients *per* year to perform a prospective study. In addition, it is very difficult to make stratification of these patients because of significant differences among them.

The most convincing examples of a different approach to empyema treatment include the use of antibiotics only [21], or repeated thoracocentesis in the treatment of complicated parapneumonic effusions and empyema [3, 22], as well as appliance of video-assisted thoracoscopic surgery in chronic empyema treatment [1, 11].

In the absence of quality research studies, recommendations for pleural infection treatment depends on pathophysiologic research, small studies and experts' opinions [23].

These circumstances were the reason for experimental empyema model development, which would be able to offer similar conditions found in human pleural infection. These models have been used for almost a century to solve the problems and controversies in empyema treatment.

Graham and Bell [13] made the first experimental empyema model in 1918 to investigate the causes of a very high mortality rate (up to 60%) among American soldieries in the World War I. They used dogs in their experiment and concluded that the open pleural drainage, prematurely performed, was the main cause of such a high mortality rate. Then they formed the Empyema Commission which introduced the closed tube underwater pleural drainage in empyema treatment, and the mortality rate dropped down to 10%.

During their work investigators came to know that any direct intrapleural application of bacteria without causing a sterile exudative pleurisy before such application would end with animal's death due to sepsis or with spontaneous healing without signs of pleural infection [24, 3].

In late 1970's, Sahn and Potts [25] made the turpentine rabbit experimental empyema model. Empyema was induced with 10^9 *K. pneumoniae* injected in the pleural space. Sterile pleurisy was provoked with 0.3 mL of turpentine 96 h before the bacterial injection.

In this paper the authors estimated the influence of administered turpentine on leucocytes number, pH, pO2 and pCO2 values in pleural exudates in the first 96 h. They revealed that in turpentine-provoked pleural effusions there was no increase of leucocytes number and bacteria growth which are responsible for metabolic activity. That was why the values of estimated the parameters did not exceed the normal range. It was very important to find that chemical injury of mesothelial cells did not affect parameters which we used for empyema classification. This model was used for research of pharmacological characteristics and therapeutic efficacy of gentamicin [15]. Turpentine rabbit experimental empyema model with 10^{10}/mL *Escherichia coli* was applied for the research of clarithromycin efficacy, newer quinolones and azithromycin penetration in the empyema fluid [9, 26, 27]. This model was also used to study linezolid and ertapenem pharmacokinetics in parapneumonic effusions [28]. This model has been proved as a better one compared with our model with two bacteria. What is next? We will try to create experimental empyema model with 10^{10}/mL *Escherichia coli*, because there is 10 times more bacteria than we accepted as optimal concentration.

The pig experimental empyema model had used an umbilical cord instead of the turpentine in order to provoke sterile effusion. Mavreudis et al. [29] applied this model to prove that the possibility for empyema induction depends on the number and strain of administered bacteria. In the case of application of *B. fragilis* bacteria, no animal developed empyema, but in combination with *S. aureus* or *E. coli,* one third of animals developed empyema. One million bacteria caused empyema in half of all animals, but with a hundred million bacteria, all animals suffered from empyema. considering these results, we decided to take 9 times more bacteria in a single (2 mL) intrapleural inoculums (4.5×10^8/mL) *S. aureus* [9].

A similar research was conducted with the rabbit turpentine empyema model [30]. The authors have studied how particular bacteria influence the severity and evolution of empyema. They found that the same number (10^8) of administered bacteria *H. influenze*, *S. aureus and B. fragilis* caused empyema in one third, one half and two thirds of animals, respectively. A combination with two bacteria was more successful in empyema induction and 11 of 12 animals evolved empyema.

Taking experience from our previous experiment when we used only *S. aureus* in intrapleural injection and gained more than one third of negative results, we decided to make a new experimental empyema model using two bacteria: *S. aureus and E. coli*. With the intention to make a greater chance for empyema induction we applied again 9 times more bacteria (1.5 Mc Farland/ml bacterial concentration) than it was recommended for this bacterial combination. Despite this fact we succeeded to induce empyema only in 8 of 12 experimental animals.

Sasse et al. [24] published their negative experiences in empyema induction by application of a monoculture bacterial specimen upon previously applied different cofactors such as mineral oil, *E. coli* supernatant, nutrition agar and talc. These authors published that pH value in their turpentine model effusion was under 7.1 only within the first 6 h, and after 24 h it rose to 7.25. The glucose level was 45 mg/dL only in the first 24 h and after that exceeded 100 mg/dL.

We got better results using the turpentine model with two bacteria compared with the above-mentioned data. In our experimental model the pH value 48 h after bacteria administration was 7.10, and 7.22 after 72 h. The glucose level was 1.23 mmol/L 24 h after we administered bacteria in pleural space, and 3.23 mmol/L after 48 hours. Glucose value returned in the normal range (4.1–5.9 mmol/L) 72 h after bacteria administration and was 4.15 mmol/L.

A positive correlation found between a growing number of leukocytes and LDH values could be explained with intense cellular destruction in an acute inflammation.

Also, with the growth of leukocytes count a glucose level became lower, which was in concordance with the results of other authors [4]. This might be explained with phagocytosis and intense bacterial metabolism which was followed by a growing intensity of glycolysis. In those conditions a bigger production of acid metabolic products was present so the measured values of pleural fluid pH were the lowest on the first day, when the intensity of inflammation was obviously the highest, and after that they became gradually bigger.

Other authors [4] using the turpentine model with *Streptococcus pneumoniae* determined the level of glucose, the value of LDH and the number of leucocytes in pleural effusion samples. The values of glucose they found were under 1.4 mmol/L at 72 h after bacteria administration, whereas the highest number of leucocytes was 5.95×10^9/L. The numbers of leucocytes that we found in effusion samples were 35×10^9/L at 24 h and 5.62×10^9/L at 72 h after administered bacterial inoculums.

In their latest rabbit experimental empyema model Sasse et al. [24] used nutritive brain heart infusion (BHI) agar aimed at keeping bacteria inside the pleural space without prior causing of chemical pleurisy. In this experiment they administered 10^8 *Pasteurella multocida*, a very virulent rabbit pathogen. Using this model the authors studied thoracocentesis effectiveness and its possibility to replace thoracic drainage in the empyema treatment, and the importance of timing for thoracic drainage to improve drainage efficacy [3, 14]. This model was also used for studying antibiotics' effectiveness by measuring their concentration in the pleural space after the parenteral administration was conducted [9, 15, 31]. On this model Sasse et al. [24] studied the role of TGF- β in the process of pleural fibrosis. These authors documented that the level of TGF-β1 correlates with microscopic thickness and the number of fibroblast in visceral pleura. This research suggests that TGF-β1 inhibition can reduce the intensity of residual pleural fibrosis.

In this empyema model the authors pH values lower than 7.1 in the samples collected 24, 48, 72 and 96 h after bacteria administration. Glucose level obtained in the effusion sample 48 h after bacteria injection was 43 mg/dL, a little above the empyema level (40 mg/dL). After 72 h, glucose values came into normal range. The glucose values obtained in this experimental empyema model were very similar to those we got in our empyema model.

After they sacrificed experimental animals, they found a dense purulent content, as well as fibrin adhesions within all pleural spaces of experimental rabbits. The rabbits from the control group did not have any intrapleural pathological changes.

We found purulent exudates and fibrin adhesions in 5 of 8 rabbits. Three rabbits had as dominant fibrin adhesions with a very little turbid pleural effusion. Two control rabbits also had no intrapleural pathological findings.

In this experimental model modification the authors placed a chest tube in the pleural space and then fixed it in the subcutaneous tissue of the scapular region, using the drain for the substances application and taking pleural fluid samples. Using this model of experimental empyema Zhu et al. [19] compared efficacy of the separate and common use of recombined human deoxyribonuclease and tissue plazminogen activator in the treatment of fibropurulent stage of empyema. We did not fix the drain in the subcutaneous tissue due to possible formation of fibrin septa that could block the discharge of pleural fluid samples.

Moxifloxacin efficiency research can serve as evidence of the turpentine model reliability. Twenty six rabbits were used for turpentine empyema model with *Streptococcus pneumoniae* and another 26 rabbits for empyema model with *Pasteurella multocida* in nutritive agar. In the results analysis after moxifloxacin administration they did not make differences between these 2 models of experimental empyema [31].

Despite many attempts to provoke experimental empyema by using *P. multocida* we did not succeed because the rabbits were dying even after inoculation of $l0^5$ bacteria. Bacteria application in solution of BHI agar did not result in the desired effect of keeping bacteria in the pleural space as all animals died with the signs of sepsis.

What is next? Rabbit experimental empyema model with *P. multocida* in BHI agar is one of the most efficient models and we will try to establish it again, now with new standardised bacterial cultures.

In the last 10 years empyema research has become basic again. Studying the role of cytokines, the powerful mediators of inflammation and tissue reparation, lowered these researches to the cellular and molecular levels.

Researches have not continued with the expected intensity and the present situation leaves enough room to continue this work the accomplishments of which would be implemented in practice in the area of the empyema treatment.

The fact that without these reliable experimental empyema models these researches would not be feasible accentuates their necessity and importance. In particular, we have been interested for the influence of some cytokines on the development of pleural fibrosis and possibilities of their inhibition. For these researches we created not so efficient rabbit experimental empyema model with *S. aureus*.

We plan to investigate the influence of TGF-β on inflammatory-proliferative processes and angiogenesis and complex cytokines network in the purulent pleural infection. Because of that we made our own model with two bacteria. Unfortunately we are not entirely satisfied with the results.

What are the next steps? The third answer to this question would be creation of the rat experimental empyema model.

Conclusion

With the intention to obtain a reliable empyemic model with more intensive pleural reaction we created the model with two different human pathogen bacteria and used 9 times higher number of bacteria than recommended in published papers. The combination of two human pathogens (*E. coli and S. aurous)* has already been proven as very effective in experimental emphysema causing. We generated acceptable, but not satisfactory results. They were a little better than in our experiment with monobacterial empyema, but not as good as we expected and came across in published respectable articles. We did not succeed in provoking an empyema in 40% of experimental animals. Obviously, the number of bacteria and bacterial combination of human pathogens are not sufficiently reliable. We plan to continue our research concerning cytokines network and angiogenesis in pleural inflammation and we need a better and more reliable experimental empyema model. Our previous experiences justify new decision making.

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