Standardization of a method of prolonged thoracic surgery and mechanical ventilation in rats to evaluate local and systemic inflammation[1]

Padronização de um modelo de cirurgia torácica prolongada e ventilação mecânica em ratos para avaliação inflamatória local e sistêmica

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ABSTRACT
Purpose: To evaluate the immediate pulmonary and systemic inflammatory response after a long-term operative period. **Methods**: Wistar rats in the experimental group were anaesthetized and submitted to tracheostomy, thoracotomy and remained on mechanical ventilation during three hours. Control animals were not submitted to the operative protocol. The following parameters have been evaluated: pulmonary myeloperoxidase activity, pulmonary serum protein extravasation, lung wet/dry weight ratio and measurement of levels of cytokines in serum. **Results**: Operated animals exhibited significantly lower serum protein extravasation in lungs compared with control animals. The lung wet/dry weight ratio and myeloperoxidase activity did not differ between groups. Serum cytokines IL-1ß, TNF-α, and IL-10 levels were not detected in groups, whereas IL-6 was detected only in operated animals. **Conclusion**: The experimental mechanical ventilation in rats with a prolonged surgical time did not produce significant local and systemic inflammatory changes and permit to evaluate others procedures in thoracic surgery.
Key words: Models, Animal. Thoracic Surgery. Inflammation. Lung. Rats.

RESUMO
Objetivo: Investigar a resposta inflamatória pulmonar e sistêmica imediata após longo período operatório. **Métodos**: Ratos Wistar do grupo experimental foram anestesiados e submetidos à traqueostomia, toracotomia e permaneceram em ventilação mecânica por três horas. O grupo controle não foi submetido ao protocolo operatório. Os seguintes parâmetros foram avaliados: atividade da mieloperoxidase pulmonar, níveis de extravasamento de proteínas séricas pulmonares, relação peso pulmonar úmido/seco e medidas dos níveis séricos de citocinas. **Resultados**: Os animais operados apresentaram menor extravasamento de proteínas séricas nos pulmões comparados aos animais controle. A relação peso úmido/seco e a atividade de mieloperoxidase não diferiram entre os grupos. As citocinas séricas IL-1ß, TNF-α e IL-10 não foram quantificáveis nos grupos, enquanto que IL-6 só foi detectada no soro dos animais operados. **Conclusão**: O modelo experimental de ventilação mecânica em ratos com tempo cirúrgico prolongado não apresentou alterações inflamatórias locais e sistêmicas significantes, permitindo avaliar a resposta inflamatória em outros procedimentos da cirurgia torácica.
Descritores: Modelos Animais. Cirurgia Torácica. Inflamação. Pulmão. Ratos.

Introduction

Human thoracic surgeries cause inflammatory responses of great magnitude and difficult management, representing a post-operative challenge to surgeons. Oxidative stress, changes in the pro and anti-inflammatory cytokines balance, ischemia-reperfusion injury, one-lung ventilation effects[1], handling of large neoplastic lesions and the surgical procedure itself are the outstanding elements that lead to endothelial dysfunction, responsible for acute inflammatory reaction.

The magnitude of the inflammatory response has a cumulative character and depends on many factors. The knowledge of each factor and preventive actions to reduce its intensity are important in clinical practice. However, ethical questions along with reproducible and comparable methods greatly difficult the human intervention studies. Therefore, the experimental surgery is an alternative to answer important questions daily observed in clinical practice[2]. To date, there is no alternative yet to replace the animal model to yield information about of pathophysiology of acute lung injury or to test therapeutic interventions in complex biological systems[2]. Certainly, animal models for human diseases cannot be more than an approximation of the human conditions[3], and caution must be taken. However, knowing the specific characteristics of the animal species used and correctly interpreting the findings it is possible to obtain key elements to elucidate lung injury in humans[2]. The literature reports experimental models in small animals that are standard for surgical procedures of short duration, but standardization procedures for prolonged operative times has been poorly explored. Therefore, this study aimed to standardize a method of long-term thoracic surgery in rats in an attempt to approximate as much as possible the human practices. We have measured the immediate post-operative pulmonary and systemic inflammatory response in anaesthetized rats undergoing thoracic surgery with mechanical ventilation.

Methods

The present study was approved by the Animals Ethics Committee of the University of Campinas (CEEA-IB-UNICAMP, protocol number: 1609-1), and followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Twenty-five male Wistar rats, weighing between 305 and 455 g, were randomly divided into two groups: Experimental (EG, n=13) and Control (CG, n=12).

All the EG rats were induced to anesthesia with isoflurane 5% through a calibrated vaporizer (Marcovap IS 707, manufactured by Scientific Instruments Narcosul LTDA - Porto Alegre, Brazil). After achieving an adequate anesthesia (verified by absence of palpebral, plantar and tail reflexes), the surgical protocol was initiated.

The animals were placed on a heated blanket. Initially, animals were tracheotomized and immediately connected to the Inter3 mechanical ventilator (Intermed® Sao Paulo, Brazil). The choice of this equipment considered the efficient adaptation with the vaporizer calibrated for isoflurane, and the possibility of working with low-flow and high respiratory rate (RR). A humidifier was also used to ensure the heating of administered gases to the animal, preventing obstructive tracheal secretion formation.

The ventilation standardized parameters used throughout the surgical protocol were: inspiratory pressure (IP) of 20 cmH_2O, positive end expiratory pressure (PEEP) of 2 cmH_2O, ratio inspiration/expiration (I:E) of 1:3, with inspired oxygen fraction (FiO_2) of 60% and RR of 80 breaths/min.

After connecting to the mechanical ventilator, the isoflurane anesthetic concentration was decreased to 1.5%, remaining in this concentration throughout the protocol. Then, the left femoral vein was dissected and used for neuromuscular blocker (pancuronium bromide) administration *in bolus* (1 mg/kg.h). The same vein was used to administer 0.9% saline solution (1 mL/h) for fluid replacement during the experiment.

The left carotid artery was cannulated and maintained with 0.9% heparinized saline (5 UI/mL) for monitoring mean arterial blood pressure (MAP) using the MacLab/400 and the Software PowerLab® (ADInstruments, NSW, Australia). Immediately after the artery cannulation, we collected a blood sample for gasometric analysis. The following gasometric parameters were evaluated: pH, PaO_2, $PaCO_2$, HCO_3-, BE and SO_2.

Right paraesternal thoracotomy with anterolateral ipsilateral extension was performed, simulating a surgical exploration necessary to detect the animal hemodynamics during the operative procedures in the thoracic cavity. Next, albumin labeled with radioactive iodine (^{125}I) was injected through the femoral vein for subsequent evaluation of pulmonary serum protein extravasation.

With the chest opened, rats remained on mechanical ventilation for 3 h, and every 15 min MAP and temperature (which remained between 37 ± 0.8ºC) for individual control were checked. During this time, lungs were kept moist with periodic applications of warm saline and the incision was covered to minimize evaporative losses. In the final minute of ventilation, a new arterial blood collection to gas analysis was performed.

At the end of the experiment, the rats received a higher dose of isoflurane. Laparotomy was then performed, and the rat was bled by the abdominal aorta. This blood was processed and used for serum cytokine analysis.

The protocol in control rats (CG) involved anesthesia induction with isoflurane just for injection of ^{125}I albumin via penile vein. After a brief recovery period of anesthesia, the animals were returned to their cages. Three h thereafter, all rats were anesthetized with isoflurane and sacrificed by exanguination similarly to EG rats.

Before removing the heart-lung block from the thoracic cavity, the lungs of all animals were perfused with 40 mL of 0.9% saline solution from a height of 20 cm through a small incision in the right ventricle, and cannulation of the pulmonar artery, followed by opening the left atrium for effluent drainage was done.

After this procedure, lungs were excised and separately to measurement of MPO activity, extravasation of pulmonary serum protein leakage and lung wet/dry weight ratio.

Pulmonary serum protein extravasation

Pulmonary serum protein extravasation was measured by means of intravenously injected ^{125}I serum albumin (2.5 μCi/kg) accumulation[4]. The radioactivity present in blood samples and lung tissue was quantified in a γ-counter. The serum extravasation was expressed as the volume (in microlitres) of serum accumulated in the lung derived from the total count in 1mL serum.

Lung myeloperoxidase activity

The right lung was removed and placed in a test tube in the presence of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (pH 6.0). The tissue sample was homogenized and centrifuged for 2 min at 14,000 rpm, and the supernatant collected. Myeloperoxidase (MPO) activity assay was performed using a microplate spectrophotometer (Spectra Max 34; Molecular Devices, Sunnyvale, CA, USA). Briefly, the assay consisted of mixing a 5 μL sample with 200 mL of o-dianisidine solution (0.167 mg/mL o-dianisidine dihydrochoride; 0.0005% hydrogen peroxide) prior to reading the plate. The changes in absorbance were measured at 460 nm recorded at intervals of 15 sec during 10 min. MPO activity was expressed as units of enzyme activity per milligram of tissue (UMPO)/mg. One unit of MPO was defined the enzyme amount that degrades one micromole of peroxide/min at 25ºC[5].

Determination of serum cytokines

Serum levels of TNF-α, IL-1ß, IL-6 and IL-10 were measured using commercially available ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Statistical analysis

Statistical analysis was performed using the software Stata 9.2. All variables were tested for normal distribution using Shapiro-Wilks W-test. When normally distributed (parametric data), data were presented as mean ± standard error of mean (SEM), applying the Student t-test. Nonparametric data were analyzed using Mann-Whitney test, and are presented as median and interquartile range (p 25-75). For intra-group comparison of nonparametric variables (MPO, extravasation of serum proteins, pH, $PaCO_2$, PaO_2, BE and SO_2) Wilcoxon test was used. Differences were considered statistically significant for all analysis if $p<0.05$.

Results

Pulmonary serum protein extravasation

Figure 1 shows that pulmonary serum protein extravasation in EG did not significantly change between right and left lungs (p=0.196). On the other hand, the protein extravasation was significantly lower in both right (p=0.014) and left lungs (p=0.039) when compared with CG.

In CG was observed increased leakage values on the right side (p=0.041), with analysis performed by Wilcoxon test.

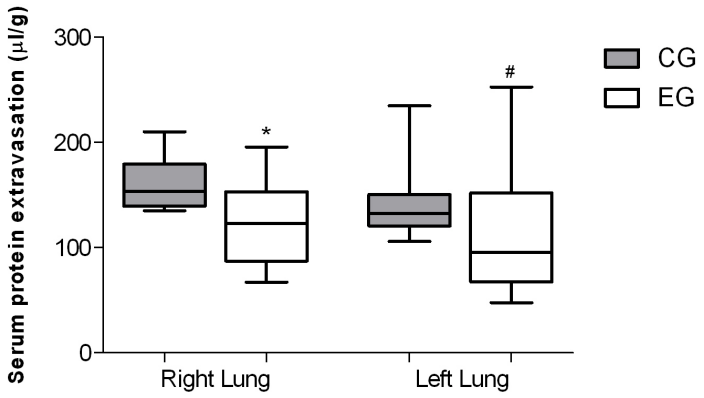

FIGURE 1 - Pulmonary serum protein extravasation in right and left lungs of control (CG) and operated rats (EG). Data represent median and interquartile range (p 25-75) for CG (n=12) and EG (n=13). *p=0.014 and #p=0.039 (Mann-Whitney test P) compared with respective CG.

The lung wet/dry weight ratio revealed no significant differences between groups (p=0.081 for the right lung and p=0.478 for the left lung).

MPO activity as a marker for pulmonary neutrophil sequestration

The MPO activity did not significantly differ between any studied groups (Figure 2). No significant differences were also found when intra-group analysis (right and left lungs) were compared.

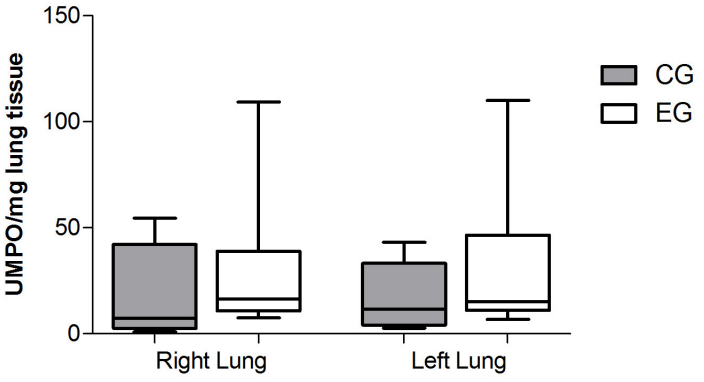

FIGURE 2 - Measurement of MPO activity in right and left lung tissue of control (CG) and operated rats (EG). There were no significant differences (Mann-Whitney test P) in the right lung (p=0.092) and left lung (p=0.211; n=12-13 each group).

Markers of systemic inflammation

The levels of IL-1ß, TNF-α and IL-10 in serum in both groups (CG and EG) were below the limit of detection, as evaluated by ELISA kit (62.5pg/mL).

The cytokine IL-6 was detected only at EG, with mean value of 1.11 ± 0.5 ng/mL.

Gasometric and hemodynamic behavior

The initial and final blood gas measurements did not differ significantly in any parameter (pH, PaO_2, $PaCO_2$, HCO_3-, BE and SO_2), as presented in Table 1. With respect to the MAP, operated group animals showed 114 mmHg as average value throughout the experiment, and the average variation between the highest and lowest MAP recorded values was 38.6 mmHg. No animal showed hemodynamic instability throughout the surgical protocol, and all of them tolerated the established ventilation time.

TABLE 1 - Initial and final gasometric values in operated rats.

	Initial			**Final**		
	Median	Percentile 25%	Percentile 75%	Median	Percentile 25%	Percentile 75%
pH	7.39	7.36	7.48	7.37	7.28	7.48
PaO_2 (mmHg)	211.8	204.4	228.7	214.7	169.1	227.3
$PaCO_2$ (mmHg)	41.2	33	47	36.7	26.6	57
HCO_3^- (mmol/L)	25.2	24.4	26.1	21.3	19.7	25.7
BE (mmol/L)	0.2	-0.7	1.9	-1.9	-4.1	-1.7
SO_2 (%)	99.6	99.6	99.7	99.6	99.1	99.6

Data presented as median and interquartile range (p 25 – 75).

Discussion

Animal models provide homogeneous, reproductive and comparable samples, which are not always achieved in clinical trials[3]. In rats specifically, animals are easily manipulated and allow procedures that would be difficult to perform in mice[6]. National literature reports the use of small animals under prolonged ventilation times, but no study exists evaluating the impact of prolonged ventilation and anesthetic times in animals undergoing surgical procedures.

Our purpose was to standardize a rat model that incorporate general anesthesia, invasive mechanical ventilation, thoracic manipulative procedures and prolonged operative time that produces no pulmonary inflammation itself (evaluated by MPO activity measurement and serum protein extravasation) and minimal impact in hemodynamic and gasometry.

Our choice for pressure-controlled ventilation took into consideration the reduction of barotrauma risk, and the best ventilation/perfusion ratio (V/Q) when compared with the volume-controlled ventilation[7].

The anesthetic agent isoflurane to induce hyperdynamic[8] instead of depressing response, proved to be a good option for the surgical procedures with long duration. The use of volatile anesthetics was initially questioned, because of its potential influences in the target-organ on the inflammatory parameters. The lung, which is the absorption route of isoflurane, would be exposed to its action; however, previous studies rather show a protective effect of volatile anesthetics in pulmonary lesions, such as those induced by ischemia-reperfusion[9]. Besides not causing lung inflammation itself in rats, isoflurane is currently employed in clinical practice.

It is well-known that blood pressure varies during surgery that may be due to changes in the central nervous system, cardiac or respiratory, with the anesthesia contributing to this disturbance[10]. After anesthesia induction and intubation, there is an initial period with minimum stimulation that is often associated with hypotension, followed by periods of intense stimulation (as evidenced in the skin incision and sternotomy), which may lead to tachycardia and hypertension[11]. Anesthetic agents should be used appropriately in anticipation of these events[11]. In our study, a stable hemodynamic response was achieved, as characterized by the absence of hemodynamic fluctuations from beginning to end of the experiment. We thus achieved an appropriate anesthetic-surgical procedure that is not usually seen with other agents such as urethane, thiopental and the mixture of ketamine and xylasine. Our gasometric data confirms the good conditions of our experimental procedures, provided no alterations in pH and gas analysis, excluding changes

in acid-base status which disturbing many enzymatic functions in biological systems[12] occurs under the experimental conditions we employed.

We also decided to evaluate the pulmonary inflammatory conditions of rats since they were under the influence of thoracotomy and mechanical ventilation. Increased permeability in the inflamed area results in loss of fluid and plasma proteins into tissues[13]. Our data showed that pulmonary serum extravasation was lower in animal undergoing mechanical ventilation with positive pressure compared with control rats. We believe this phenomenon is a consequence of the pulmonary physiology and ventilatory mechanics. The mechanical ventilation with positive pressure alters the cardiac performance when compared to spontaneous breathing[14]. Under physiological breathing, the negative pressure phase during the inspiration favors venous return, relieves pressure on the pulmonary capillaries and stimulates the flow; in contrast, with the positive pressure ventilation, intrathoracic pressure increases during inspiration causing a decrease in venous return, in right ventricular output and in pulmonary blood flow[15]. At expiration, the venous return increases; however, if PEEP is employed, the continuous positive intrathoracic pressure inhibits venous return also during expiration[15]. This may explain the lower serum protein leakage seen in rats undergoing positive pressure through the ventilatory cycle. PEEP was likely to be responsible for increasing the interstitial pressure, reducing the pressure gradient between the vascular and interstitial space[16]. In clinical practice, the use of positive pressure is routinely used since moderate degrees of PEEP may limit the pulmonary edema formation[16]. Furthermore, exposure of thoracic cavity to atmospheric pressure after thoracotomy influences the strength of extra-alveolar vessels representing one more influential factor on the lung perfusion in the operated animals group. The protein extravasation response was not observed when using the wet/dry lung weight analysis. The lower specificity of the gravimetric evaluation (wet/dry weight) may explain this finding.

Neutrophils are prominent in inflammation, because they migrate directed by chemotactic gradients into the injured site and release the contents of their granules, which play a central role in the early inflammatory response[17]. The MPO present in azurophilic granules of neutrophils generates oxygen reactive species, enhances the cytotoxic state and contributes to inflammatory response[18]. The MPO enzymatic activity can be used as a marker for neutrophils sequestration[19]. In this work, the MPO activity did not differ significantly between groups, reinforcing the low grade of injury caused by surgical and ventilatory technique in the immediate postoperative evaluation. Moreover, we did not detect significant amounts of TNF-α and IL-1ß (along with the absence of the IL-10) in serum of rats undergoing thoracotomy and mechanical ventilation. TNF-α and IL-1ß are well-recognized to cause endothelial cell damage and hence increased permeability, and induce neutrophil transmigration through the vascular endothelial membrane[14], whereas the anti-inflammatory IL-10 is present under inflammatory conditions, yielding clinical protection and reduction of lung pathology[20]. We may not neglect that volatile anesthetics act negatively modulating the inflammatory response (and TNF-α release) as demonstrated in experimental models of ischemia and reperfusion[9]. Furthermore, this study detected IL-6 serum only in animals undergoing surgery. An important finding to consider is that although systemically expressed, this cytokine did not promote the neutrophils recruitment to lung tissue in GE animals as demonstrated through the absence of significant difference in MPO activity between the studied groups.

Lastly, zero mortality index was detected in our study, which allows us to support that, after several preliminary studies, we develop a biologically correct model for inflammation study in lung surgical procedures with long anesthetic-surgical duration.

Conclusion

The experimental mechanical ventilation in rats with a prolonged surgical time did not produce significant local and systemic inflammatory changes and permit to evaluate others procedures in thoracic surgery.

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