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METHYLENE BLUE ATTENUATES ISCHEMIA-REPERFUSION INJURY IN LUNG TRANSPLANTATION

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Marcus da Matta Abreu and Rogério Pazetti – designed the study, performed the surgeries, interpreted the data and wrote the manuscript

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ABSTRACT

Background: Ischemia-reperfusion injury is one of the principal obstacles for the lung transplantation success. Several strategies have been adopted to minimize the effects of ischemia-reperfusion injury in lungs, including ex vivo conditioning of the grafts and the use of antioxidant drugs, such as methylene blue. We hypothesized that methylene blue could minimize the effects of ischemia-reperfusion injury in a lung transplantation rodent model. **Methods:** Forty rats were divided into four groups (n=10) according to treatment (saline solution or methylene blue) and graft cold ischemic time (3 or 6 h). All animals underwent unilateral lung transplantation. Recipients received 2 mL of saline or methylene blue intraperitoneally before transplantation. After 2 h of reperfusion, arterial blood and exhaled nitric oxide samples were collected and bronchoalveolar lavage performed. Then animals were euthanized and histopathology analysis, as well as cell counts and cytokine levels measurements in bronchoalveolar lavage fluid were performed. **Results:** There was a significant decrease in exhaled nitric oxide, neutrophils, IL-6 and TNF-α in methylene blue-treated animals. PaO_2 and uric acid levels were higher in methylene blue group. **Conclusion:** Methylene blue was able in attenuating ischemia-reperfusion injury in this lung transplantation model.

Keywords: animal model, ischemia-reperfusion injury, methylene blue, lung transplantation

1. Introduction

Lung transplantation (LTx) is a well-established therapeutic option for the treatment of end-stage lung diseases such as chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis, bronchiectasis, and primary pulmonary hypertension[1]. However, ischemia-reperfusion injury (IRI) remains one of the principal obstacles for LTx success. In its severe clinical presentation, IRI is known as primary graft dysfunction (PGD), which is associated with high morbidity and mortality in the first days after LTx[2].

A PGD is a situation that involves complex cellular, molecular and biochemical changes. Several mechanisms contribute at the same time to the formation of morphological and functional changes that are characterized by an increase in pulmonary vascular resistance, increased pulmonary capillary permeability and edema, leading to an impaired gas exchange with an increase in the alveolar-arterial oxygen gradient and a decrease in PaO_2. The morphological response of the endothelium is characterized by the presence of apoptosis and infiltration by macrophages and polymorphonuclear cells. The hypoxia leads to an increased expression of cell adhesion molecules and the production of Reactive Oxygen Species (ROS) leading to an activation of microvascular endothelial cells that become even more dysfunctional. At the same time, the pulmonary surfactant undergoes changes in its composition, function and metabolism, leading to a reduction of lung compliance[3].

The etiology of PGD primarily involves the increased formation of reactive oxygen species (ROS)[1]. Briefly, a decreased oxygen supply reduces the synthesis and resynthesis of adenosine triphosphate (ATP), creating an ionic gradient in the cell membrane due to decreased extracellular active calcium transport. The accumulation of cytoplasmic calcium leads to the activation of a protease that converts xanthine dehydrogenase to xanthine oxidase[3]. Concurrent with these events, there is an accumulation of adenosine monophosphate (AMP), which decomposes into substances such as adenosine, inosine, and hypoxanthine. During reperfusion

process, in the presence of oxygen, xanthine oxidase converts hypoxanthine into ROS such as superoxide, peroxide, and hydroxyl radicals[3,4].

Several strategies have been adopted to minimize the effects of IRI in lungs, including ex vivo conditioning of the grafts and the use of antioxidant drugs, in both clinical and experimental settings[5,6]. Some studies have recently investigated the antioxidant properties of methylene blue (MB)[7,8]. MB has been used successfully as inhibitory drug in lung lesions caused by intestinal ischemia followed by reperfusion in rats[4] and in the treatment of secondary hemodynamic changes to reperfusion in liver transplantation[9] . MB prevents ROS production by acting as an alternative xanthine oxidase electron receptor, competing with molecular oxygen for electron transfer[4]. The electrons are transferred to MB from the iron-sulfur center of xanthine oxidase, thus preventing the conversion of molecular oxygen into superoxide[4].

Another action mechanism related to MB and the IR process is the inhibitory action on Nitric Oxide (NO). The inducible nitric oxyde synthase (iNOS), which is expressed through the action of inflammatory mediators, can give rise to radical peroxynitrite and peroxynitrous acid, which participate in lipid peroxidation processes and increased endothelial cell adhesion. In the lungs, NO can lead to the formation of toxic peroxynitrite, resulting in an increased inflammatory response[3].

Our aim was to evaluate the effects of MB as an inhibitor of IRI in rats after LTx.

2. Material and Methods

Eighty female Sprague-Dawley rats (300-350g) were used in this study (40 donor/40 recipients). Recipient rats were divided into four groups (n=10) according to graft cold ischemic time of 3 or 6 h, and treatment with saline solution (SAL) or MB: 3SAL, 6SAL, 3MB, and 6MB. This study was approved by our institutional research committee (CAPPesq 3387/09/138) and performed according to the Guide for the Care and Use of Laboratory Animals [10].

2.1 Surgical procedure

2.1.1 Donor

Animals were anesthetized with isoflurane 5% (Isothane, Baxter, Porto Rico, USA), orotracheally intubated and mechanically ventilated (model 683, Harvard Apparatus, Holliston, MA, USA) with 10 mL/kg and 80 cycles/min. General anesthesia was maintained with isoflurane 2% (isovapor mod. 1224, K. Takaoka, Brazil). After median laparotomy, 500 U of heparin was injected into inferior vena cava. After one minute, a median sternotomy was performed and pulmonary artery cannulated for anterograde perfusion with 20mL of low-potassium dextran solution (LPD) (Perfadex®, Vitrolife, Sweden) at 4°C with constant pressure (20 cm H_2O). Prior to perfusion, inferior vena cava was sectioned to decrease venous return, and the left atrial appendage was amputated to drain the LPD. Animals were euthanized by exsanguination, according to the Report of the American Veterinary Medicine Association Panel on Euthanasia [11].

After perfusion, trachea was tied at the end of inspiratory flow and the cardiopulmonary block was excised and placed in a Petri dish with cold LPD for back table step. Left hilum was dissected and cuffs fixed in the artery, vein, and bronchus, as previously described [12]. Grafts were maintained inflated during ischemia period (3 or 6 h) and were stored in cold LPD till implantation.

2.1.2 Recipient

Recipient animals were anesthetized, intubated and ventilated as described above. Immediately prior to graft implantation, animals were intraperitoneally injected with 2 mL of either SAL 0.9% or MB 1%. Then they were placed in right lateral recumbence and subjected to left thoracotomy at the fourth intercostal space. Subsequently, graft implantation was performed using a stereomicroscope (model SZ61, Olympus, Tokyo, Japan) at 8x magnification[12]. In brief, left hilum was dissected and clamped as proximal as possible. Then, graft implantation was performed by introducing graft cuffs into a little hole made in ventral wall of the artery,

vein and bronchus, respectively. After cuffs fixation using a 7.0 polypropylene silk, bronchus clamp was slowly opened and air flow reestablished. In sequence, vein clamp was removed for retrograde circulation establishment and, finally, artery clamp was gently opened aiming a soft graft perfusion. The closure of the recipient incision was performed in separate layers using 2.0 monofilament nylon sutures. After surgery completion, animals received analgesia (dipyrone 400 mg/kg) by gavage and were placed under spontaneous ventilation in individual cages with free access to water and food.

2.2 Exhaled nitric oxide (eNO)

After 2h of graft reperfusion, animals were anesthetized, intubated and ventilated as previously described. Under these conditions, eNO was obtained using a Mylar balloon connected to the ventilator expiratory way for 3 minutes. To avoid environmental contamination, a zero NO filter (Sievers Instruments, Inc.) was attached at the inspiratory valve of the ventilator; eNO levels were measured by chemiluminescence (NO analyzer, NOA 280, Sievers Instruments, Inc., Boulder, CO). Just before each measurement, the NOA was calibrated with NO gas at 47 ppb[13].

2.3 PaO_2 and uric acid analysis

After eNO test, a laparotomy was performed and blood samples were collected from the abdominal aorta (0.3 mL) and inferior vena cava (1.0 mL) for PaO_2 and uric acid analysis, respectively. During collection, the animals were ventilated with a 100% FiO2.

Then animals were euthanized by severing abdominal aorta and cardiopulmonary block was excised from thoracic cavity.

2.4 Bronchoalveolar lavage fluid (BALF)

BALF was obtained by tracheal instillation of 3 mL of cold phosphate-buffered saline (PBS), which was centrifuged at 1,000 rpm for 10 min at 5°C. After centrifugation, TNF-α, IL-6, and cinc-1 were measured in BALF supernatant by ELISA (DUO SET kit, R&D System®, CA,

USA). Cell pellet was resuspended with 5 mL of PBS for total cell count in Neubauer chamber. Subsequently, cytospin slides were prepared (Cytospin-2 model, Shandon Instruments, Sewickley, PA) and stained with Diff-Quik for differential cell count (300 cells/slide).

2.5 Histological analysis

Left lung was fixed by tracheal instillation of formaldehyde solution 4% (20 cm H_2O) and stored for 24h in the same solution for histological analysis. Paraffin-embedded lung samples were cut into 5-µm sections and stained with hematoxylin and eosin. Point-counting histomorphometry was applied to quantify inflammatory cells, edema and hemorrhage in lung parenchyma by using a Weibel grid containing 100 points and 50 lines. Ten random and mismatched microscopic fields were examined with 400x magnification, totaling 1,000 points per slide and covering an area of 62,500 μm^2 per field.

2.6 Statistical analysis

Descriptive analysis was performed for quantitative data with normal distribution, expressing the results as mean and standard deviation (M±SD). Normality of data distribution and homogeneity of variances were evaluated by Shapiro-Wilk and Levene tests, respectively. Two way ANOVA test was used for dependent quantitative variables. A 0.05 probability of Type I error (α) was considered for all inferential analysis.

3. Results

3.1 PaO_2, uric acid and eNO

PaO_2 was higher in 3MB (150.2 ± 50.1 mmHg) in comparison with 3SAL (102.6 ± 40.4 mmHg, p=0.028) (Figure 1). eNO was lower in 3MB (3.2 ± 2.0 ppb, p=0.05) (Figure 2). Uric acid levels were higher in MB-treated animals (3MB= 4.7 ± 0.9 mg/mL, 6MB= 5.3 ± 2.4 mg/mL) when compared to those from SAL groups (3SAL= 2.7 ± 0.7 mg/mL, p=0.003; 6SAL= 2.3 ± 0.9 mg/mL, p<0.001) (Figure 3).

3.2 Inflammatory cells and cytokines in BALF

The number of neutrophils in BALF was lower in MB groups, but with significance only at 6h of ischemia (3MB=21.9 ± 12.1 $X10^4$ vs 3SAL=33.5 ± 17.9 $X10^4$, p=0.138; 6MB=11.8 ± 7.4 X 10^4 vs 6SAL=30 ± 19.2 X 10^4, p=0.023) (Figure 4). In its turn, IL-6 was lower in MB groups at both ischemia times (3MB=122.4 ± 24.9 pg/mL, p=0.008; 6MB=142 ± 38.7 pg/mL, p=0.002) (Figure 5a). However, TNF-α was lower just in 6MB when compared to 6SAL (189.5 ± 93.5 pg/mL, p=0.007) (Figure 5b). There was no difference among groups concerning total cell count, other cell types and cinc-1 in BALF.

3.3 Inflammatory cells, edema and hemorrhage in lung parenchyma

The neutrophil count in perialveolar tissues was higher in 6SAL group (5.1 ± 3.1 %, p=0.046). (Figure 6). Perivascular edema was higher in 6SAL group in comparison with 6MB group (35.2 ± 7.6 %, p=0.001) (Figure 7a). Edema in the perialveolar tissues was higher in 3SAL group (28.1 ± 18.2 %, p=0.041), (Figure 7b). There was no difference between groups concerning intra-alveolar hemorrhage (3MB=11.9 ± 5.6 %, 3SAL=20.7 ± 11.5 %, p=0.39; 6MB=15.7± 7.0 %, 6SAL=26.4 ± 13.0 % ; p=0.10).

There was no difference between MB and SAL groups with respect to neutrophil count in the perivascular tissues (Figure 5a). In addition, there was no difference in the counts of plasmocytes, macrophages, eosinophils or lymphocytes in both perivascular and perialveolar tissues.

4. Discussion

IRI remains one of the principal causes of graft loss after LTx. IRI mechanism is complex and several studies have been performed aiming to avoid or at least to attenuate its effects. Our results show that MB administration to recipient animals prior to LTx attenuates IRI by reducing neutrophil migration, TNF-α and IL-6 expression, and perivascular edema.

Compared to other anti-oxidizing agents, MB has as advantage that it is a low cost drug that has been used for over a century with infrequent adverse effects, and that it is only contraindicated in cases of renal failure and hypersensitivity[14]. It has been used for the treatment of systemic inflammatory response syndrome in the postoperative of thoracic surgery[15], in the treatment of hemodynamic changes after reperfusion injury in liver transplantation[9] and vasoplegic syndrome after cardiovascular surgery with cardiopulmonar bypass[7,8]. The beneficial effects of MB in Alzheimer's disease and its cerebral protection in animals submitted to cardiorespiratory arrest have ben studied[16,17].

IRI after LTx is characterized by increased microvascular permeability and the pulmonary sequestration of polymorphonuclear cells. Reperfusion of the ischemic lung leads to the greater adhesion of these cells to the endothelium and to lung injury mediated by polymorphonuclear leukocytes[18]. We found a significant lower number of neutrophils in MB-treated animals in both BALF and perialveolar tissue.

The degree of pulmonary edema is inversely proportional to the quality of lung preservation[19]. Several experimental models have used edema measurement to evaluate the efficacy of protective drugs against IRI after LTx[20,21]. Our results showed a decreased edema in perivascular and perialveolar tissues of MB-treated rats.

In the present study, a PaO_2 measurement was taken after 120 min of graft reperfusion, and the difference was evident only for the groups subjected to 3-h ischemia. Xu et al.[22], using a model of pulmonary IR in rabbits, showed a rapid functional recovery of the animals, suggesting that measurements at earlier reperfusion times in our study may have shown more pronounced differences for both ischemic periods.

In addition, the absence of exclusion of the native lung may have influenced these results. We chose to adopt this experimental model, with the arterial blood being harvested with both lungs functioning, to mimic what occurs in transplants in human beings [23] .

In LTx, the eNO level is an excellent marker of airway inflammation[24] and increased exhaled oxide nitric levels in rodents submitted to a lung transplantation reflect acute rejection[25]. The animals in the MB group subjected to 3-h ischemia showed significantly lower eNO levels than the SAL group; the same difference was not observed in the animals subjected to 6-h ischemia. These results are consistent with a lower degree of airway inflammation in the 3MB group.

TNF-α acts as a mediator of acute lung injury and the induction of edema[26,27]. The findings in the present study, such as the reduced levels of TNF-α, correlate with the reduced inflammatory infiltrate identified in the BALF and the histological findings with regard to inflammation and edema.

Sotoudeh et al.[28] studied an experimental model in which N-acetylcysteine was used to prevent IR-induced lung injury in skeletal muscle. The histological analysis of the lungs showed greater edema, alveolar hemorrhage, and neutrophil infiltration in control group animals. The present study also observed greater edema and neutrophil infiltration in the control group animals; however, there was no decrease in alveolar hemorrhage in animals treated with MB. This result may occur because in our model, there is lung manipulation and bleeding secondary to trauma as a result of the LTx.

ROS lead to the activation of alveolar macrophages, which in turn stimulate the production of inflammatory cytokines that can react with nitric oxide and lead to the formation of highly toxic radicals known as reactive nitrogen species (RNS)[3]. Without the action of xanthine oxidase, hypoxanthine would be transformed into uric acid[3].

The measurement of uric acid as a method for indirect quantification of xanthine oxidase activity was proposed by Greca et al.[4]. Kelner et al.[29]proposed the use of MB as a blocker of ROS production in the IR process and hypothesized that MB accelerates the conversion of hypoxanthine into uric acid. In addition, Den Hengst et al.[3] considered that MB favors the uric acid formation by inhibiting xanthine oxidase action and then accelerating hypoxanthine degradation. This suggests that elevated serum levels of uric acid may characterize a beneficial

molecular mechanism in the context of IRI, which corroborate the increased uric acid levels in MB groups in our study.

In the present study, IL-6 levels were decreased in the MB group at both ischemic durations. Because this interleukin participates in inflammation by modulating several aspects of the inflammatory process, including cell proliferation, its blockage by MB is concordant with the decreased inflammatory response observed in the present study, as demonstrated by the reduction of neutrophils in BALF and in lung parenchyma.

As a limitation of our study, we can highlight that we did not induce brain death in the donor animals. As such, we did not replicate the clinical pulmonary transplantation model exactly. In addition, we used the same dose of methylene blue in all cases. This drug has a dose-dependent effect, and a study with different doses could produce different results than those found here. We chose to employ the same dose for all surgical procedures (approximately 50 mg/Kg) as in a previous study[4], in which the dose was also administered intraperitoneally and proved to be effective in protecting the lungs against the deleterious effects of intestinal IR.

In addition, the monitoring of hemodynamic parameters, markers of tissue perfusion and ventilation parameters could be useful for a better understanding of the action of MB. Future work in this line of research should meet this need.

MB has an important effect on vasoplegic phenomena[7,8], such as those that occur as a result of IR lesions[9]. Despite the importance of combating vasoplegia by attempting to restore the vascular tonus, doses "above the ideal" can lead to a worsening of tissue perfusion, generating an increase in anaerobic metabolism and acidotic state[30]. We therefore also believe that studies involving the analysis of microcirculation may clarify the dose-dependent effect of MB. We also think that further studies evaluating the dose-response effect of MB in similar models to the one used here, can help guide us to the "ideal" dose range to be used.

The present model for evaluating IR lesions in experimental LTx in rats has proven satisfactory regarding the times chosen for ischemia and reperfusion of the grafts. The effects of reperfusion occur early, as shown in a previous study[31].

5. Conclusion

The present study demonstrates for the first time that MB is an effective drug for protection against IR-caused injuries following LTx in rats, leading to decreased inflammation and edema formation. The complex physiopathology of this event associated with the absence of a drug capable of achieving the aforementioned goals makes us believe that experimental models using multiple substances with different properties against the IRI process should be tested to achieve a greater chance of LTx success.

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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- We hypothesized that MB could minimize the effects of IRI in a LTx rodent model.
- The etiology of PGD primarily involves the increased formation of ROS
- MB prevents ROS production
- MB was able in attenuating IR injury in this lung transplantation model.

Figure 1: PaO_2 in groups with 3-h ischemia (p=0.028) and 6-h ischemia (p=0.472). MB: Methylene Blue; SAL: Saline.

Figure 2: eNO levels in groups with 3-h (p=0.05) and 6-h ischemia (p=0.713). MB: Methylene Blue; SAL: Saline.

Figure 3: Uric acid measurement in groups with 3-h (p=0.003) and 6-h (p<0.001) ischemia. MB: Methylene Blue; SAL: Saline.

Figure 4: Neutrophil count in BALF fluid for groups with 3-h (p=0.138) and 6-h ischemia (p=0.023). MB: Methylene Blue; SAL: Saline.

Figure 5: Cytokine quantification in BALF in groups with 3-h and 6-h ischemia. a: IL-6 (3 h, p=0.008; 6 h, p=0.002); b: TNF-α (3 h, p=0.710; 6 h, p=0.007). MB: Methylene Blue; SAL: Saline.

Figure 6: Neutrophil count with 3-h and 6-h ischemia. Perialveolar tissues (3 h, p=0.135; 6 h, p=0.046). MB: Methylene Blue; SAL: Saline.

Figure 7: Edema quantification from 3-h and 6-h ischemia. a: perivascular tissues (3 h, p=0.083; 6 h, p=0.001); b: perialveolar tissues (3 h, p=0.041; 6 h, p=0.074). p<0.05. MB: Methylene Blue; SAL: Saline.

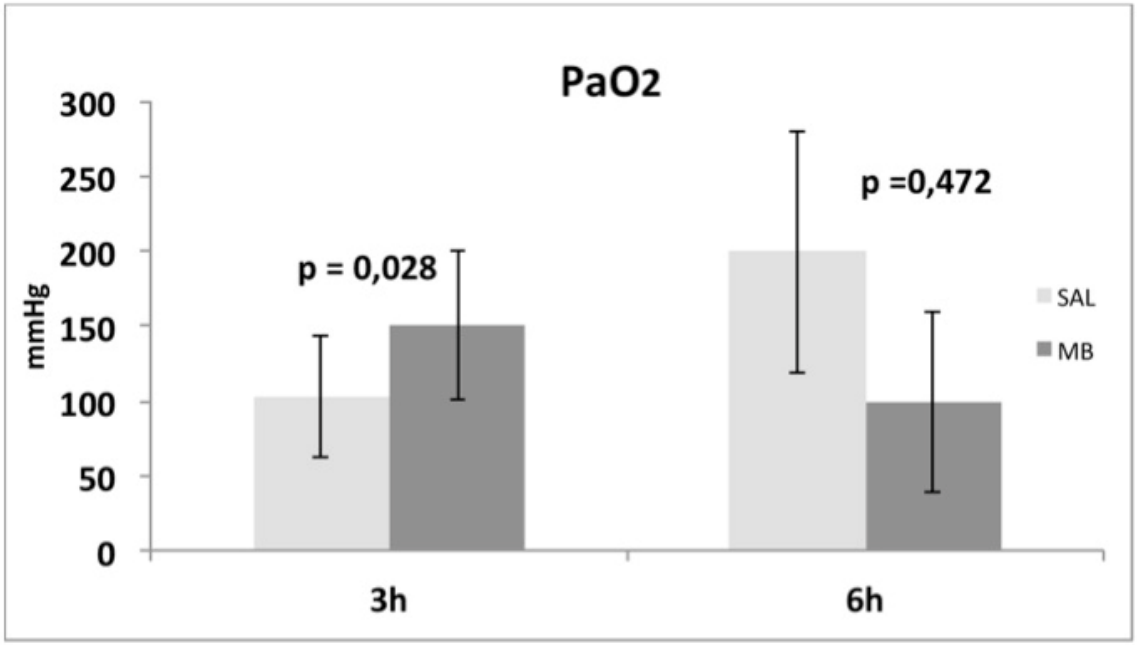

Figure 1

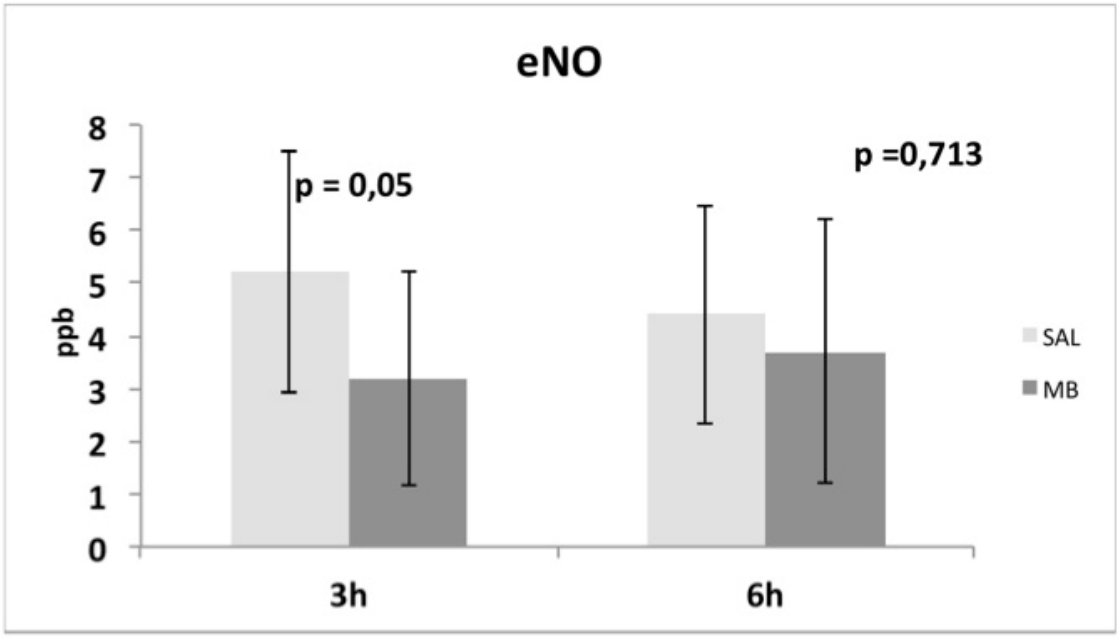

Figure 2

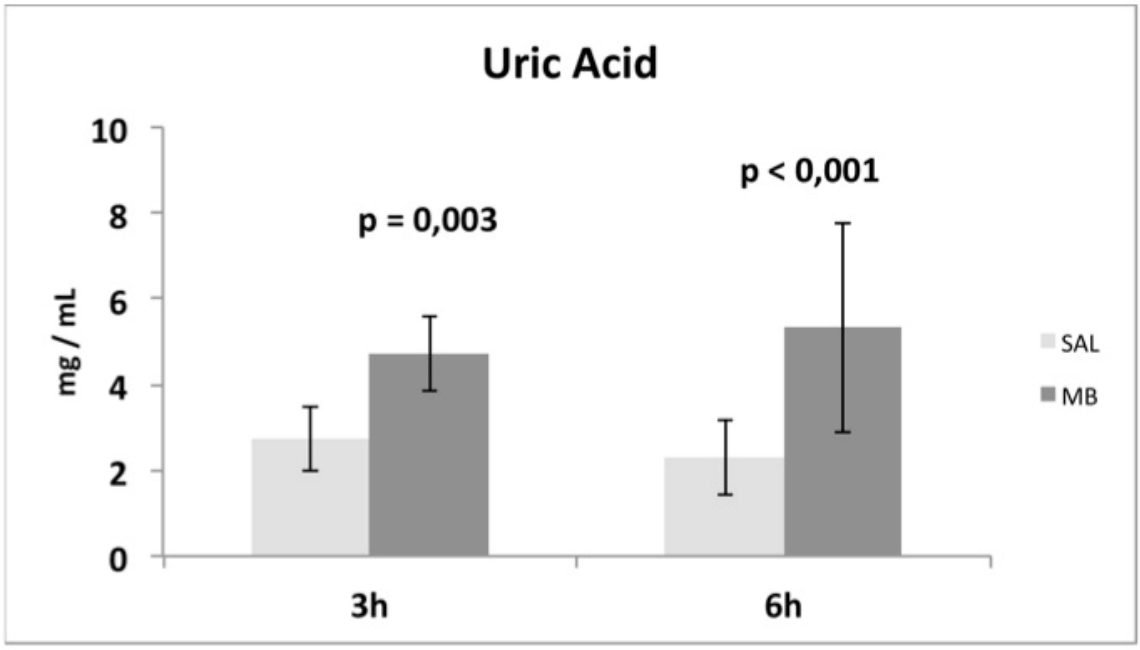

Figure 3

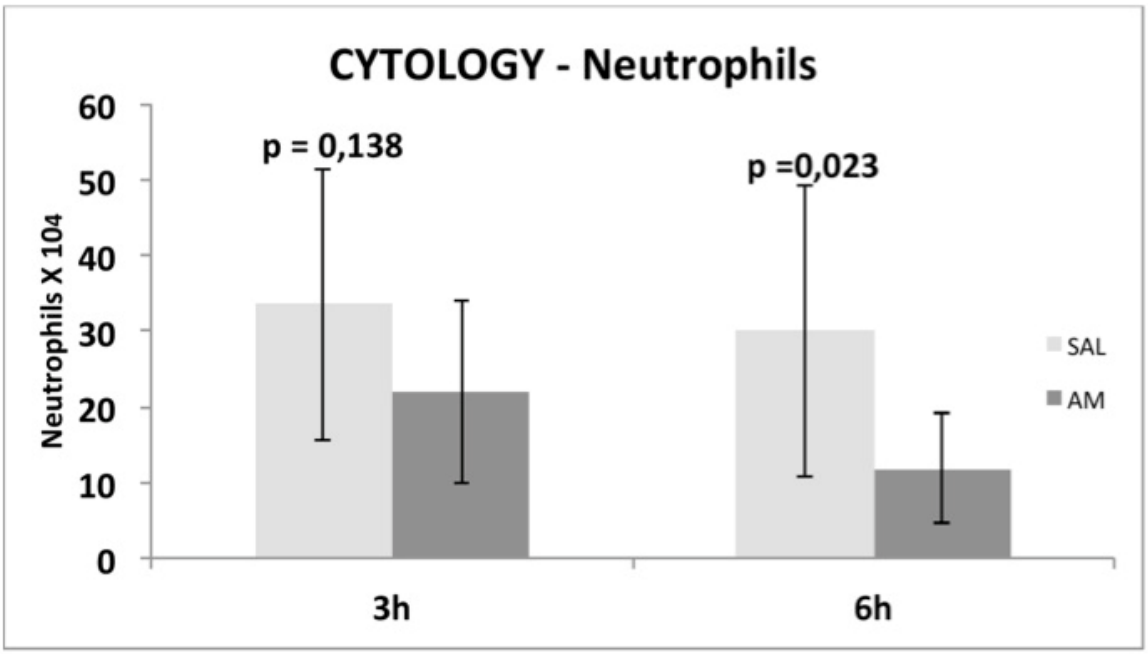

Figure 4

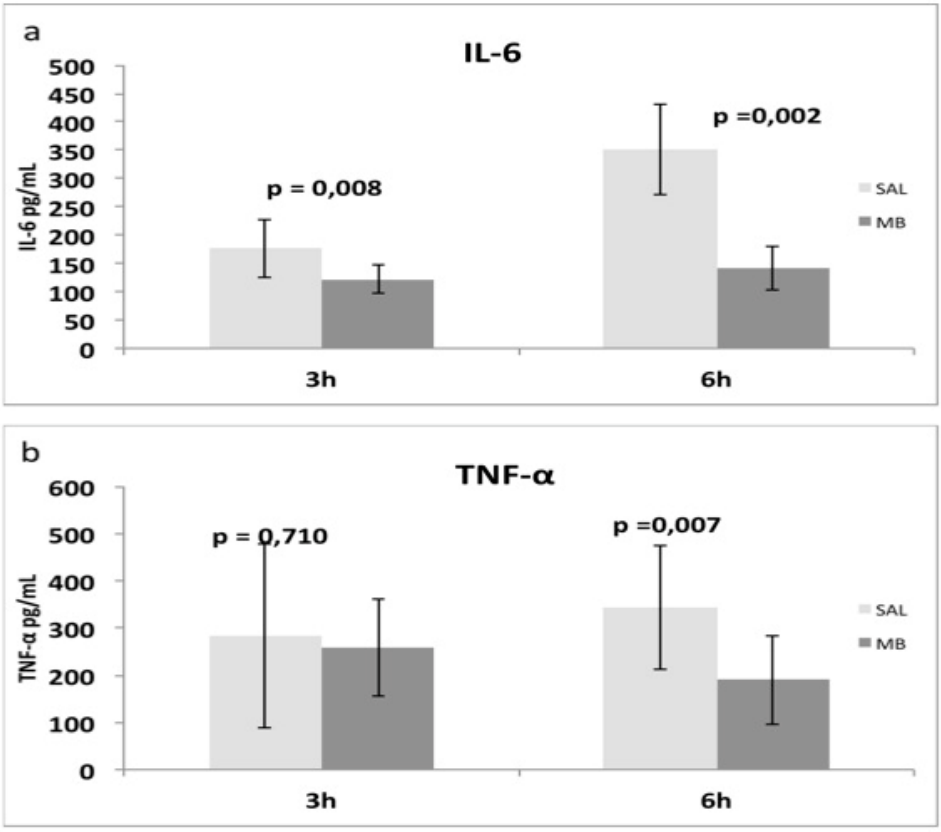

Figure 5

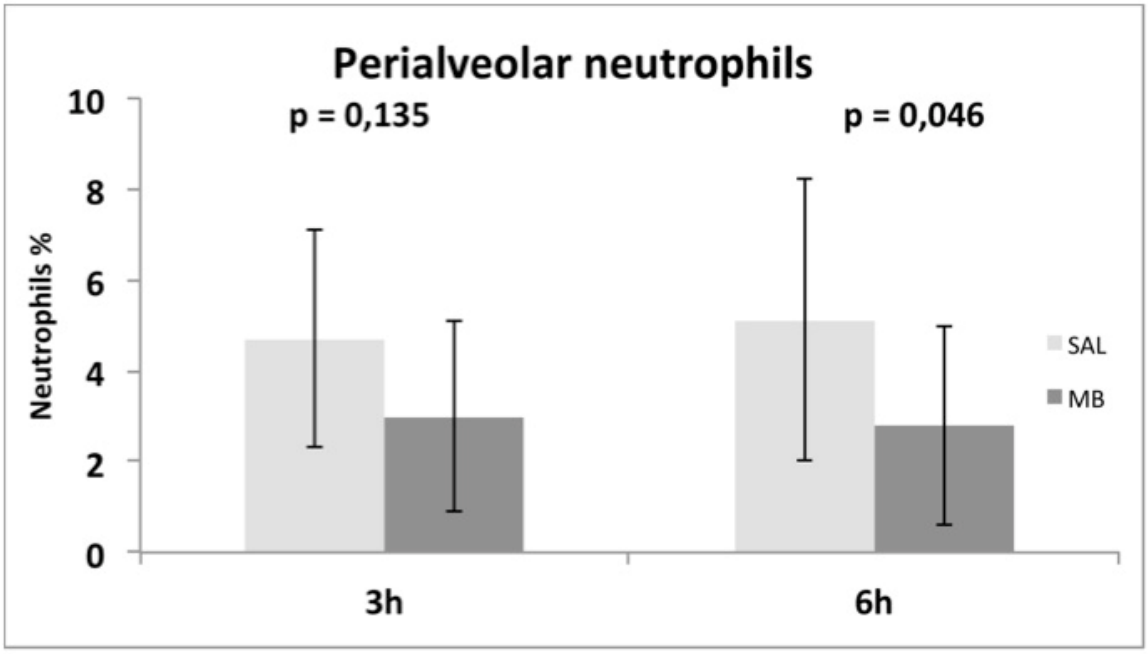

Figure 6

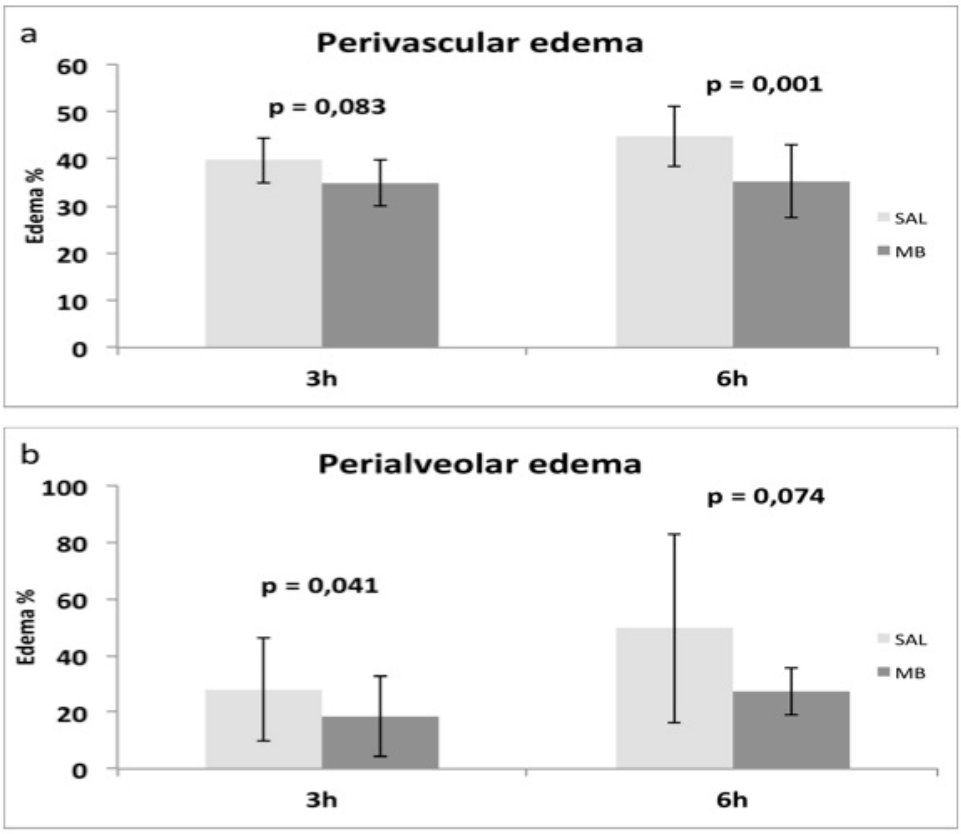

Figure 7