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Pulmonary impact of N-acetylcysteine in a controlled hemorrhagic shock model in rats

Karen Ruggeri Saad, PhD,[a] Paulo Fernandes Saad, MD, PhD,[a] Luiz Dantas Filho, MD, PhD,[a] Jôse Mára de Brito, PhD,[b] Marcia Kiyomi Koike, PhD,[c] Fernando Luiz Zanoni, PhD,[d] Marisa Dolhnikoff, MD, PhD,[b] and Edna Frasson de Souza Montero, MD, PhD[a,e,]*

[a] *Department of Surgery, Medical School, Federal University of São Paulo, São Paulo, Brazil*
[b] *Department of Pathology, Medical School, University of São Paulo, São Paulo, Brazil*
[c] *Laboratory of Clinical Emergencies (LIM-51), Medical School, University of São Paulo, São Paulo, Brazil*
[d] *Cardiovascular Surgery and Circulation Physiopathology Laboratory, Medical School, University of São Paulo, São Paulo, Brazil*
[e] *Surgical Physiopathology Laboratory (LIM-62), Medical School, University of São Paulo, São Paulo, Brazil*

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ABSTRACT

Background: Experimental hemorrhagic shock (HS) is based on controlling bleeding and the treatment of fluid resuscitation to restore tissue oxygenation and perfusion. The HS could promote ischemia/reperfusion injury, which induces a general exacerbation of the inflammatory process, initially compromising the lungs. N-acetylcysteine (NAC), an antioxidant, may attenuate ischemia/reperfusion injury. This study evaluated the effect of NAC in association with fluid resuscitation on pulmonary injury in a controlled HS model in rats.

Methods: Male Wistar rats were submitted to controlled HS (mean arterial pressure of 35 mm Hg for 60 min). Two groups were constituted according to resuscitation solution administered: RLG (Ringer's lactate solution) and RLG+NAC (Ringer's lactate in association with 150 mg/kg NAC. A control group was submitted to catheterization only. After 120 min of resuscitation, bronchoalveolar lavage was performed to assess intra-alveolar cell infiltration and pulmonary tissue was collected for assessment of malondialdehyde, interleukin 6, and interleukin 10 and histopathology.

Results: Compared with the RLG group, the RLG+NAC group showed lower bronchoalveolar lavage inflammatory cell numbers, lower interstitial inflammatory infiltration in pulmonary parenchyma, and lower malondialdehyde concentration. However, tissue cytokine (interleukin 6 and interleukin 10) expression levels were similar.

Conclusion: N-acetylcysteine was associated with fluid resuscitation–attenuated oxidative stress and inflammatory cell infiltration in pulmonary parenchyma. N-acetylcysteine did not modify cytokine expression.

1. Introduction

Hemorrhage is responsible for approximately 50% of deaths in trauma and also contributes to late mortality—death 48 h after injury [1]. Interventions in hemorrhagic shock (HS), such as fast bleeding control and fluid resuscitation, are fundamental to restore tissue perfusion and oxygenation, reducing morbidity and mortality in trauma. However, this life-saving

* *Corresponding author*. Alameda Espada, 134-Res Onze, Alphaville, ZIP: 06540-395 Santana de Parnaíba – SP, Brazil. Tel.: +55 11 3061 7216 or +55 11 9686 7933.
E-mail address: edna.montero@gmail.com (E.F.S. Montero).
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procedure may induce ischemia/reperfusion (I/R) injury and, eventually, compromise vital organs [2,3].

Ischemia/reperfusion injury is a potent trigger of the inflammatory process, since it generates reactive oxygen species (ROS), activating endothelial cells, consequently leading to nitric oxide production and adhesion molecule expression and increasing cytokine production [4]. These events initiate pulmonary disorders characterized by accumulation of alveolar and interstitial fluids, alveolar hemorrhage, fibrin deposition, and neutrophil sequestration [5]. Neutrophil accumulation in the interstitial and alveolar space is considered the critical event in the pathophysiological process of acute lung injury (ALI). Acute respiratory distress syndrome, the most severe form of ALI, is associated with increased morbidity and mortality. The mechanism by which I/R modulates immunity, leading to exacerbated pulmonary response, is not completely understood, but studies show that ALI occurs after reperfusion, with fast generation of ROS and cytokines [6,7].

The oxidative stress caused by I/R [8] promotes increase in membrane lipid peroxidation, which could be measured by malondialdehyde (MDA) or thiobarbituric acid reaction. The produced ROS lead to systemic effects, activating circulating neutrophils as well as recruiting neutrophils to the pulmonary parenchyma. Cytokines produced by neutrophils, such as interleukin 6 (IL-6), are chemoattractive to alveolar macrophages and endothelial cells, causing an exacerbation of inflammatory response and ALI. In experimental models of I/R, despite increased production of proinflammatory cytokines, there is production of antiinflammatory cytokines, such as interleukin 10 (IL-10), a regulatory mechanism in inflammatory response [9].

It has been demonstrated that antioxidant agents, when administered during resuscitation, play a role in reducing oxidative stress by two distinct mechanisms: (1) removing ROS; and (2) interfering in the cell signaling process, consequently reducing inflammatory response [3,10–13].

N-acetylcysteine (NAC) is a low-cost, highly available substance with a low level of adverse effects. It is metabolized into cysteine, a precursor of glutathione, which in its reduced and oxidized forms participates together with glutathione peroxidase in the ROS degradation cascade, acting in removing tissue free radicals [14].

There is evidence that NAC may protect the lungs in I/R injury in experimental models of lung [15–17] and liver [18–20] transplantations. However, the effect of NAC on pulmonary parenchyma in HS is poorly studied. For this reason, we evaluated the effect of NAC on ALI, when combined with fluid resuscitation in rats submitted to HS.

2. Materials and methods

2.1. Animal preparation

All animals were handled according to the ethical principles of laboratory animal care, and this study's protocol was approved by the Research Ethics Committee of the Unifesp (no. 1261/09).

Adult male Wistar rats (*Rattus norvegicus albinus*), aged between 90 and 120 d and weighing between 250 and 300 g, were used. The animals were kept in a vivarium for 6 d for observation and adaptation, where they received water and balanced food rations *ad libitum* and were kept in appropriate cages (40 × 30 × 25 cm), with a maximum of five animals per cage, under controlled conditions for light, temperature, and daily hygiene.

The animals were anesthetized with a combination of ketamine (50 mg/kg) and xylazine (15 mg/kg) via intraperitoneal injection. The animals were considered anesthetized when they were unresponsive to mechanical stimuli, being unable to withdraw the hind limb after pain stimulus and also presented absence of palpebral reflexes. Additional doses of anesthesia (half of the initial dose) were administered approximately every 50 min. The animals were also kept well ventilated at room temperature.

The animals were placed on a constantly heated plate (37°C) in supine position with hind limbs immobilized and adhesive tape across the chest. Trichotomy was performed on the right anterior cervical and right inguinal regions with a razor blade followed by antisepsis of the operative area with iodine polyvinylpyrrolidone tincture.

Using a no. 11 scalpel blade, a skin incision was made, with dissection and isolation of the right common carotid artery and incision in the right inguinal region with dissection and isolation of the femoral artery and vein. An Intracath 22 G catheter (Becton Dickinson, Sandy, UT) was used for catheterization. A venous catheter was used for injecting heparin and fluids for resuscitation according to the experimental groups, and arterial catheters were used for collecting blood samples, exsanguinations to promote the shock, and monitoring mean arterial pressure (MAP).

For MAP control and effectiveness of the procedures employed, the arterial catheter was connected to a pressure transducer connected to a calibrated preamplifier and a computerized data acquisition system (Dixtal DX 2020, DIXTAL BIOMÉDICA INDÚSTRIA E COMÉRCIO LTDA, Manaus, AM, Brazil), in which hemodynamic data were stored (MAP and heart rate). At the end of surgery, the animals were observed for 15 min for MAP stabilization.

2.2. Experimental groups and inducing hemorrhagic shock

The animals were randomly distributed into the following groups:

- Control group (CG, $n = 6$): This group of animals was only submitted to the surgical procedure and catheterization, without HS induction.
- Ringer's lactate group (RLG, $n = 6$): This group of animals was submitted to HS and treated with Ringer's lactate solution (33 mL/kg) and, soon after, 50% blood (previously withdrawn) as fluid resuscitation.
- Ringer's lactate + N-acetylcysteine group (RLG+NAC, $n = 6$): This group of animals was submitted to HS and treated with Ringer's lactate solution (33 mL/kg) associated with NAC (150 mg/kg) and, soon after, 50% blood (previously withdrawn) as fluid resuscitation.

To induce HS, nonfractionated heparin was infused (100 IU/rat) and blood was taken via the arterial catheter for

10 min, using a previously heparinized 10-mL syringe, until MAP reached 35 mm Hg; MAP was then maintained for 60 min, with removing or infusing of heparinized whole blood if MAP changed ±5 mm Hg. After this period, rats were submitted to fluid resuscitation according to their specified groups. Resuscitation was considered successful when MAP remained above 80 mm Hg for at least 5 min.

The animals were observed for 120 min, at which time they were euthanized by exsanguination while still under anesthesia.

2.3. Collecting bronchoalveolar lavage and lung tissue

After euthanasia, tracheotomy and median sternotomy were performed by clamping the left main bronchus. The right lung was washed with 2.5 mL phosphate buffer solution (PBS) at 4°C through a tracheal cannula three consecutive times, totaling 7.5 mL PBS. The amount recovered was placed into Eppendorf tubes and centrifuged at 1850 rpm for 10 min at 4°C (5804 Centrifuge; Eppendorf, Hamburg, Germany).

Next, the left lung was collected and divided into two parts by a longitudinal cut. The lateral portion was fixed in 10% formaldehyde solution and embedded in paraffin. The medial portion of the lung was immediately placed into cryogenic tubes and frozen in liquid nitrogen for biochemical assays.

2.4. Estimating lipid peroxidation

We determined cell membrane lipoperoxidation in pulmonary tissue caused by ROS by the thiobarbituric acid reactive substances method, which measures the amount of MDA derived from lipid peroxidation, expressed as nanomoles per mg of protein (nmol/mg protein).

The medial portion of the left lung was frozen at −80°C and a fragment was homogenized in 1 mL of 1.15% KCl with a sonicator (Polytron PT-MR 3100, Kinematica AG - Lucerne, Switzerland). Therefore, aliquots were centrifuged at 10,000 rpm for 20 min at 4°C (5804 Centrifuge; Eppendorf).

For the thiobarbituric acid reactive substances reaction, the following was added: 100 μL of the supernatant, 100 μL of 8.1% sodium dodecyl sulfate, 750 μL of 20% acetic acid, and 750 μL of 0.8% thiobarbituric acid. The mixture was heated for 50 min at 95°C. After the established period, 200-μL samples were analyzed in a spectrophotometer at 532 mn (Multiscan Ex; MTX Lab Systems, Vienna, VA). The results were expressed in μg/mg protein and analyzed in duplicate.

2.5. IL-6 and IL-10 measurement by ELISA

The levels of IL-6 and IL-10 were measured in pulmonary tissue using the ELISA method with the commercial antibody pairs, recombinant standards, and the biotin-streptavidin-peroxidase detection system, as described previously [21]. All reagents, samples, and working standards were brought to room temperature and prepared according to the manufacturer's directions (R & D Systems, Inc, Minneapolis, MN). Briefly, the tissue samples were homogenized in 1 mg/mL PBS and centrifuged at 2600 rpm (5804 Centrifuge; Eppendorf) for 15 min at 6°C. The supernatant collected was used for measurements. Reactions were quantified by optical density using an automated ELISA reader (Multiscan Ex; MTX Lab Systems). All analyses were performed in duplicate.

2.6. Bronchoalveolar lavage

A Neubauer chamber was used for total cell counts. The bronchoalveolar lavage (BAL) was centrifuged and the pellet was resuspended in 1000 μL PBS. The total cell count was calculated by the following formula: total cell count/mL = total counts × 10^5 × sample dilution.

For differential counting of inflammatory cells, 100 μL of resuspended solution was placed in a cytologic centrifuge (Cytospin 3 Shandon Scientific, Pittsburgh, PA) and centrifuged at 450 rpm for 6 min. The cytospin slide was stained with Diff Quick (Baxter Dade, Dudingen, Switzerland) for identification and differential counting of macrophages, neutrophils, lymphocytes, and eosinophils under an optical microscope at 100× magnification. For differential cell counts, 300 cells were considered per slide.

2.7. Morphologic analysis

Four-micrometer sections of pulmonary tissue were stained with hematoxylin–eosin. The histologic slides were qualitatively evaluated under an optical microscope (Axio Imager A2; Zeiss, Oberkochen, Germany) by an experienced pathologist kept masked to the different treatment groups. At least 20 fields of each slide were randomly chosen and analyzed. Lung injury severity was evaluated by a histologic score based on four parameters: congestion, hemorrhage, neutrophil interstitial infiltration, and lymphocyte interstitial infiltration. Each parameter was evaluated by a score using the following scale: 0, absent; 1, mild; 2, moderate; 3, severe. The total score corresponding to the inflammatory lesions was calculated by summing the values attributed to each parameter for each animal (total varying from 0–12) as previously reported [22]. Then the descriptive statistics were calculated for each group.

2.8. Statistical analysis

The data were analyzed using the SigmaStat statistical program, version 3.1 (Systat Software, San Jose, CA). The groups were compared by 1-way analysis of variance or Kruskal-Wallis 1-way analysis of variance on ranks, after testing for normality and variance equality, and complemented by *post hoc* test (Student-Newman-Keuls). Difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. Lung oxidative stress

The HS-induced groups showed significant increase in MDA levels compared with CG. In contrast, NAC treatment reduced MDA levels. Figure 1A shows MDA levels in the lung from all experimental groups.

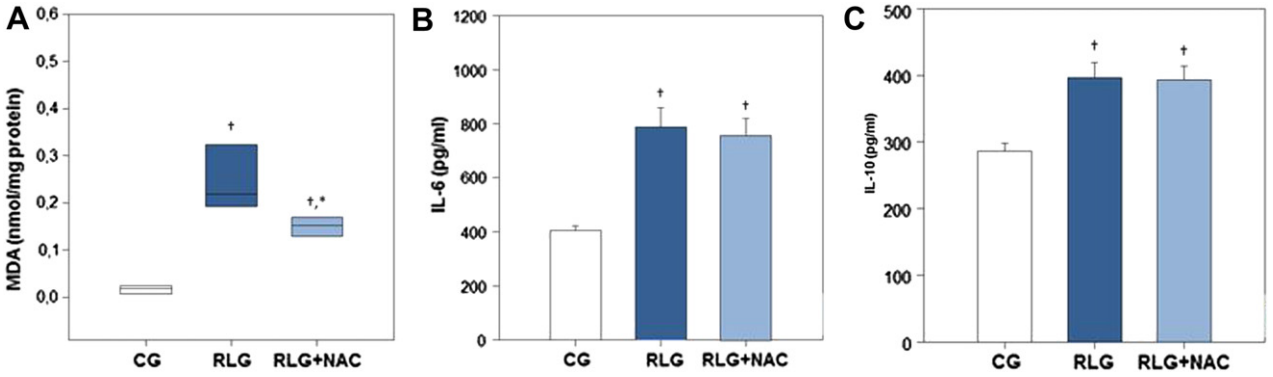

Fig. 1 – (A) Lipid peroxidation estimated by malondialdehyde level (MDA), (B) proinflammatory cytokine expression (IL-6), and (C) antiinflammatory cytokine expression (IL-10) in pulmonary parenchyma after hemorrhagic shock and resuscitation. Data are expressed as median (interquartile range) for MDA and mean ± standard deviation for cytokines. †, *versus* CG. *, *versus* RLG. (Color version of Figure is available online.)

3.2. *IL-6 and IL-10 in lung tissue*

The IL-6 and IL-10 expression levels were elevated in HS-induced groups compared with CG, but were similar between RLG and RLG+NAC. The IL-6 and IL-10 expression in the lung is shown in Figure 1B and C.

3.3. *Bronchoalveolar lavage cellularity*

The number of total cells in BAL in RLG was elevated compared with CG and RLG+NAC. The total number of cells was similar between CG and RLG+NAC. There was increased BAL cellularity in RLG due to the increased number of macrophages. Total and differential cell counts in BAL are shown in Table 1.

3.4. *Morphologic analysis*

The HS model used in this study demonstrated that lung injury is characterized by interstitial neutrophilic infiltration in absence of intra-alveolar hemorrhage. However, RLG+NAC showed reduction of lung injury compared with RLG, indicating that NAC could exert a protective action, attenuating inflammatory response. Figure 2 illustrates the results referring to pulmonary histopathologic evaluation. The morphologic changes were graded and summarized in Table 2.

Table 1 – Assessment of total and differential bronchoalveolar lavage cells.

	CG ($n = 6$)	RLG ($n = 6$)	RLG+NAC ($n = 6$)
Number of cells (cell/mL × 10^4)	3.92 ± 0.92	8.62 ± 2.73*	5.67 ± 1.69†
Macrophages (cell/mL × 10^4)	3.43 ± 0.94	8.25 ± 2.57*	4.66 ± 2.01†

Data shown as mean ± standard deviation.
* $P < 0.05$ *versus* CG.
† $P < 0.05$ *versus* RLG.

4. Discussion

Trauma patient treatment has shown significant improvement in the last four decades; however, mortality rates for ALI and acute respiratory distress syndrome remain close to 40% [23]. To date, no pharmacologic therapy has demonstrated reduced decreased mortality in clinical trials.

According to Tasoulis *et al.* [8], post–hemorrhagic shock fluid resuscitation promotes an exacerbated inflammatory response in the lungs and contributes to ALI. This injury is characterized by accumulated neutrophils in lung tissue, representing the main sources of ROS generation, adhesion molecule expression in endothelium of pulmonary capillaries, and increased cytokine expression [24,25]. In addition to ROS production by polymorphonuclear cells, development of an inflammatory cascade originating from endothelial cells present in the pulmonary parenchyma has a secondary oxidative effect that intensifies the primary damage [26].

Despite the influence of inflammatory mediators in ALI development, we emphasize that this injury's pathophysiology is complex and multifactorial, involving alterations to microcirculation, in addition to the endothelial changes previously mentioned. There is evidence that hypoperfusion of the splanchnic territory leads to inflammatory alterations in intestinal mucosa, not only promoting lung injury, but developing dysfunction in multiple organs and systems [27].

In recent years, studies have looked into drugs combined with solution for fluid resuscitation in order to attenuate inflammatory response [28,29]. Considering NAC's beneficial effects in experimental I/R models developed in this laboratory research, this study aimed to investigate the effect of NAC on lung injury due to hemorrhagic shock in rats [30].

N-acetylcysteine was developed in the 1960s for reducing disulfide bonds, and it is used extensively to reduce viscosity and elasticity in mucus [31]. NAC's antioxidant function may be explained by its molecule's formation, since the SH group has the potential to directly interact with oxidants such as H_2O_2, forming H_2O and O_2. In addition to the ROS-inactivating function, there is evidence that NAC promotes cellular glutathione production, reducing cellular and tissue damage [32].

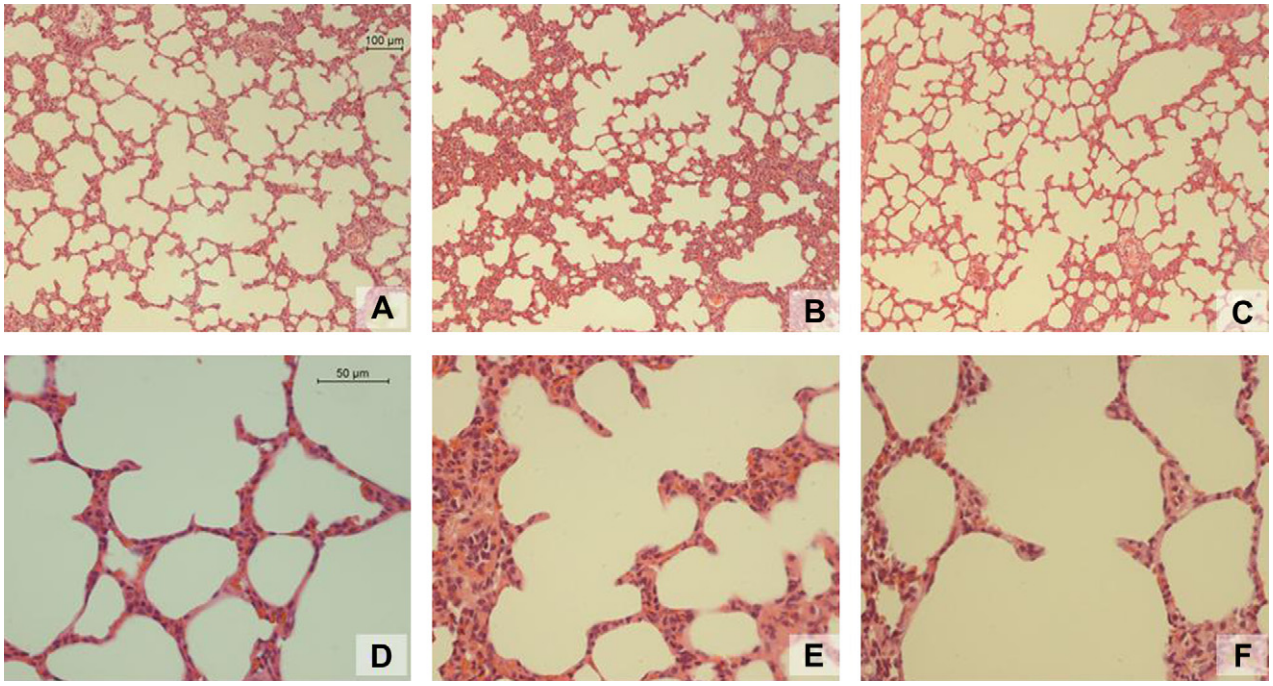

Fig. 2 – Photomicrographs of representative lung sections from the three groups, stained with hematoxylin–eosin. (A–C) Magnification ×10. Preservation of lung parenchyma structure in RLG+NAC (C) is similar to CG (A) and different from RLG (B). (D–F) Magnification ×40. Photomicrographs show increased neutrophil infiltration of the RLG (E) when compared with CG (D) and RLG+NAC (F). (Color version of Figure is available online.)

Reactive oxygen species play a crucial role in inducing lung injury; however, it is difficult to quantify these molecules because of their short half-life. Numerous assays provide indirect evidence of ROS activity, which includes measurement of consumption of endogenous antioxidant substances such as glutathione, superoxide dismutase, and catalase. Other biochemical markers of the oxidative state are the end products of lipid peroxidation, including MDA [8].

Lipid peroxidation in lung tissue observed in other studies [33–35] indicates that oxidative stress occurs during I/R. Similarly, in hemorrhagic shock models, oxidative stress was observed in lung tissue, as reflected by elevated MDA levels [36].

Inci *et al.* [16] reported significantly reduced oxidative stress in rat lungs treated with NAC after I/R injury. These authors also observed increased glutathione in lung tissue for the I/R group treated with NAC just before tissue reperfusion.

Table 2 – Morphologic analysis of pulmonary parenchyma by scores according to Deree *et al.* [22]

	CG ($n = 6$)	RLG ($n = 6$)	RLG+NAC ($n = 6$)
Hemorrhage	0	0	0
Congestion	0.5	1	1
Neutrophils infiltrated	0.5	3	2
Lymphocytes infiltrated	0	2	1
Total score	1 (0–2)	6 (6–6)*	4 (4–4)*,†

Total score data shown as median (interquartile range).
* $P < 0.05$ *versus* CG.
† $P < 0.05$ *versus* RLG.

NAC also attenuated oxidative stress in rat lungs in a model of ALI induced by administering intratracheal lipopolysaccharide [37].

In this study, the hemorrhagic shock model promoted oxidative stress, which was attenuated by NAC combined with fluid resuscitation, as shown by quantifying MDA. Another quite similar study on hemorrhagic shock models reported attenuation of hepatic oxidative stress [38].

In contrast, Alkan *et al.* [39] found no decrease in pulmonary oxidative stress in rats subjected to hemorrhagic shock and treated with NAC. However, the protocol of administering NAC may have contributed to this finding, since 80 mg/kg was orally administered for 3 d before the shock and an additional 80 mg/kg before fluid resuscitation.

In evaluating inflammatory response, we chose to quantify the IL-6 and IL-10 cytokines because they represent proinflammatory and antiinflammatory pathways in I/R lesions [40]. Studies [41,42] have demonstrated that IL-6 is produced in lungs of rats subjected to hemorrhagic shock and is located in bronchoepithelial cells and alveolar macrophages. Expression of messenger ribonucleic acid for IL-6 increases the hypovolemic shock's longer duration, but both shock and fluid resuscitation phases are involved in this cytokine's increased expression.

Expression of messenger ribonucleic acid for IL-6 appears at 60 min of initial fluid resuscitation, causing polymorphonucleated cell infiltration and lung injury characterized by congestion [41]. In this study, we observed high tissue IL-6 concentrations in groups of animals subjected to hemorrhagic shock; however, NAC was unable to decrease this cytokine's expression in lung tissue after 2 h of fluid resuscitation.

An experimental study on lung I/R models observed decreased IL-6 expression in BAL in the group of animals treated with NAC before the lung ischemia period, as well as the group of animals that received NAC before the reperfusion period, which lasted 4 h [15]. Similarly, Kim *et al.* [43], using an intestinal I/R model, observed that NAC treatment reduced IL-6 expression when administered after 1 h of intestinal ischemia and 3 h before reperfusion (between the 2 h in hypothermia), compared to a reperfusion-simulated group with 2 h in normothermia. The results of these studies indicate NAC's possible role in late IL-6 expression in lung tissue, a period that was not evaluated in our study.

It is known that IL-10 is an immunoregulatory cytokine that inhibits synthesis of proinflammatory cytokines and recruitment of polymorphonuclear cells [44]. The literature suggests that the degree of the effect of IL-10's immunomodulation mechanism may be dependent on injury type. Study using a visceral I/R model showed no differences in levels of tumor necrosis factor α and IL-6 between animals with and without IL-10 deficiency [45]; in another study, where the authors examined the modulatory effect of IL-10 on different hemorrhagic shock models, there is a trend for suppressing IL-6 in animals treated with exogenous IL-10 [46].

In this study, NAC combined with solution for fluid resuscitation did not alter IL-10 pulmonary expression, nor did it alter the direct, inversely proportional relationship with IL-6 tissue expression in both groups subjected to hemorrhagic shock. Chamogeorgakis *et al.* [47] also reported similar results in expression of IL-10 and tumor necrosis factor α in lung tissue, unmodified by NAC treatment in a lung I/R model (90 min ischemia and 180 min reperfusion). This again suggests the need for study assessing late periods combined with different NAC doses.

It is noteworthy that in BAL, NAC decreased cellularity, mainly at the expense of macrophages, despite that the IL-6 tissue expression remained unaltered. Corroborating the BAL findings was the lower histopathologic score, evidenced primarily by lower infiltration of neutrophils and lymphocytes compared with RLG. These results appear to be related to lower degrees of oxidative stress observed in NAC treatment. Another study [48] involving animal hemorrhagic shock models also observed that the amount of cellular infiltration in the pulmonary interstitium was directly related to the amount of tissue oxidative stress indicators.

In evaluating BAL cellularity, the main difference between groups was due to the number of macrophages, whereas a difference in the differential percentage of neutrophils, lymphocytes, or eosinophils between the two groups of animals subjected to hemorrhagic shock was not observed in this phase, although an increase in neutrophil infiltration was observed in the interstitium. Possibly, protocols with longer hemorrhagic shock and/or post–fluid resuscitation periods would allow for observing the second phase of ALI, dependent on infiltration and activation of neutrophils [49].

However, other studies that evaluated the effects of NAC treatment on lung injury in hemorrhagic shock or I/R lung models observed no reduced BAL cellularity or attenuation of histopathologic changes [15,16,39]. We must consider the differences in experimental protocols of these studies, mainly related to NAC doses used, in addition to pathways and stages of administration.

Limitations of this study include using a single NAC dose of 150 mg/kg combined with fluid resuscitation and an experimental protocol that allowed for observing lung injury after 1 h of hypovolemic shock and just 2 h of hemodynamic stability after fluid resuscitation. Further studies should be conducted using other NAC doses, 600 mg or 1200 mg, as well as longer experimental protocols, with up to 4 h after fluid resuscitation.

The results of this study suggest that NAC has a promising role as a drug that can be combined with fluid resuscitation in treating hemorrhagic shock. Other studies should be conducted to determine the best dose-response relationship and the best therapeutic window.

5. Conclusion

The results of this study indicate that NAC in association with fluid resuscitation after hemorrhagic shock can attenuate lung injury by reducing oxidative stress, BAL cellularity, and tissue inflammatory infiltrate.

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