UNIVERSIDADE FEDERAL DE SERGIPE
CAMPUS UNIVERSITÁRIO PROFESSOR ANTONIO GARCIA FILHO
DEPARTAMENTO DE FISIOTERAPIA DE LAGARTO

ANALYSIS OF EVOLUTIONARY PROCESS OF THE TRACHEAL AND PULMONARY INJURY BY SMOKE INHALATION IN RODENTS

DÉBORA DO NASCIMENTO SANTOS

Lagarto – SE
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Trabalho de Conclusão de Curso apresentado ao Departamento de Fisioterapia de Lagarto, Universidade Federal de Sergipe, como parte dos requisitos para graduação em Fisioterapia, sob a orientação do(a) Prof. Carlos José Oliveira de Matos.

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Lagarto, 19 de dezembro de 2019.

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SUMÁRIO

ANALYSIS OF THE EVOLUTIONARY PROCESS OF THE TRACHE AND PULMONARY INJURY BY SMOKE INHALATION IN WHEELS SUBMITTED TO OXYGENOTHERAPY.

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ABSTRACT

The aim of this study was is to analyze the evolutionary process of lung and trachea by smoke inhalation injury in rodents. The study was conducted with 15 rats' subjects to smoke inhalation through burning cotton during twenty-seven minutes inhaling smoke and one minute and thirty second inhaling air. Then, they receipted oxygen during thirty minutes. They were divided into 3 groups such as, control group (CG), group 24 hours (h) (G24), group 48 h (G48) and 72 h (G72). The animals were sacrificed 24, 48 and 72 h after the induction of inhalation injury and the CG was sacrificed in the same time of G24. Posteriorly, the tissues of the trachea and lungs were collected for analysis histological, malondialdehyde (MDA), sulfidril (SH), catalase (CAT), tumoral necrosis factor α (TNF- α), interleukin-1 β (IL-1β) analyses. The obtained results showed that smoke inhalation injury worsens the architecture of the trachea and lung due histological changes caused by intense inflammatory response (G24 and G48 of trachea and lung) triggering pulmonary edema (mainly G48 in lung) and, consequently, emphysema (G48 and G72 lung) and areas of fibrosis (G72 lung) when compared CG. Morover, the levels of TNF-α and IL-1β were highs in trachea tissue, only in G24 with p=0.01 and p=0.00, respectively, when compared to CG. Besides, we also observed that oxidative stress high in all injured groups (G24, G48 and G72) with highs concentrations of levels serums of MDA (p=0.00) when compared to CG. However, we observed lows levels of SH in all injured groups in trachea (p=0.01) compared to CG, in comparison with levels of SH in lung tissue that no obtained difference significative (p=0.402). Also, lows levels of CAT in lung (p=0.02) in G24 compared to G48, G72 and CG. In this sample, exposure to smoke induced a focal, diffuse and acute inflammatory process in tracheal tissues on the respiratory epithelium, with loss of ciliated epithelial tissue, in addition to presenting interstitial and alveolar edemas and infiltrates of inflammatory cells in the pulmonary parenchyma in animals of experimental groups. Moreover, concomitant with the above changes, in the tracheal tissue, these alterations are also related to inflammatory cytokine activities (TNF and IL-1β), which corroborates the action of oxidative stress.

Keywords: Lung injury. Smoke. Inhalation. Histology. Oxidative stress. Cytokines.

INTRODUCTION

In major fire disasters such as the Kiss Club in southern Brazil (2013), smoke inhalation caused serious complications in the victims, such as burning of the body surface, dyspnea, torpor, dizziness and even evolution to coma or death [1]. The injury smoke inhalation should deteriorate the respiratory system within a few hours and encompasses characteristics like bronchospasm, respiratory distress, alveolar injury, as well as, upper and lower edema and bacterial pneumonia [2]. This inhalation consists of an inhalation injury, which increases mortality by 24 times [3] due to the action of toxic gases, which are released though incomplete combustion and thermal degradation of the materials present at the site [4].

Inhalation injury is the reaction of the inflammatory process of the airways after uninhibited absorption of incomplete combustion products [4]. There are three physiological types of injury: (1) thermal lesion, with higher incidence in the upper airway, also consisting of a direct cellular lesion, concomitant with a significant edema, which appears in the first 24 hours; (2) chemical lesion, both in the upper and lower airways (bronchi and bronchioles), exciting the mucosa and causing intense inflammatory infiltrate, mediated by polymorphonuclear cells, mainly neutrophils, edema, with consequent reduction of surfactant and atelectasis; (3) systemic poisoning of toxic gases such as carbon monoxide (MO) and cyanide (CN) [3,5].

From the moment the injury process begins, the organism activates a complex antioxidant protection system in order to defend itself against free radicals – formed in normal cellular metabolism and pathological process – and, when in excess may cause the oxidation of biological molecules. Thus, oxidative stress (OS) is defined as an imbalance between oxidizing and antioxidant systems. Therefore, oxidative stress markers, such as malondialdehyde, appear increased, evidencing stress through altered levels of antioxidants, such as catalase and sulfhydryl. [6].

The process of smoke inhalation injury is influenced by factors such as temperature, type of material burned and room ventilation. In the consequent inflammatory process, the main inflammatory mediators released are: tumor necrosis factor alfa (TNF-α), interleukin 8 (IL-8) – powerful chemotactic agent and neutrophil activator, interleukin 6 (IL-6), interleukin 1β (IL-1β), kappa nuclear factor β (NF-kβ), malondialdehyde (MDA), myeloperoxidase (MPO) and catalase (CAT) [7,8].

Considering the risks of smoke inhalation, as well as its negative impact on lung function and hemodynamic stability, the Research Group on Burns, of the Federal University of Sergipe (UFS - SE, Brazil) has been researching, since 2014, inhalation injuries from smoke

inhalation. The rodent model used by researchers De Carvalho (2016) and Silva (2018), also based on studies by Li (2013), Qiu [9] and Melo (2011), describe the structural, functional and immunohistochemical changes in the respiratory system of rats submitted to this type of injury. Thus, the purpose of this research was to analyze the evolutionary process of lung injury and trachea of rodents after smoke inhalation.

MATERIAL AND METHODS

This is an experimental, longitudinal, randomized study, approved by the Ethics Committee for the Use of Animals (CEUA) of University Federal of Sergipe, approval record 73/2018. Were included twenty male rats, Wistar, of approximately 90 days of life and body mass around 250 and 350 grams, provided by the Bioterium of Morphology/Physiology of the Universidade Federal de Sergipe were included in this study. The preference for the use of the small rodents is due to their easy manipulation, low cost and good reproducibility with the human model, especially in relation to hematological, histological and immunological analyses [1, 9].

The rats were divided into four groups with different times of sacrifice. Three groups were submitted to the same inhalation injury model by burning cotton for a total of 27 minutes, followed by exposure to ambient air for 1 minute and 30 seconds in total. After the moment of injury, the rats waited 30 minutes and after that they received 30 minutes of 100% oxygen inhalation. Five of them were sacrificed within 24 hours (G24), another five in the 48h group (G48) and the others in the 72h group (G72). [1, 11, 13].

A priori, the twenty rats were exposed to smoke inhalation, one at a time, produced by 30g of cotton per body weight of the animal (30g/kg). The inhalation system employed included an air chamber measuring 40.5 cm x 35 cm x 23 cm, respectively length x width x height. The rats were exposed to successive periods of 09 minutes of smoke inhalation followed by 30 seconds of exposure to ambient air. This process was repeated three times according to the studies of Qiu [9], which totaled 28’30” (twenty-eight minutes and thirty seconds), of which 27’ were injuries and 1’30” of exposure to ambient air [1, 13].

After 30 minutes of induction of inhalation injury, the rats waited 30 minutes, spontaneously ventilating at room temperature, simulating the waiting time for help to arrive, and after that all of 15 rats inhaled 100% oxygen. The promotion of hyperoxia, as recommended by experts, aimed to reduce carboxyhemoglobin half-life by up to six times [5]. Subsequently, after completing the estimated time of experimentation, five animals were sacrificed in group 24 hours (G24), after group 48 hours (G48) were sacrificed five ones, and group 72 hours (G72)

more five ones. They were weighed and anesthetized for sacrifice. Ketamine (80-90 mg/kg intraperitoneal weight) and Xylazine (2% - 10 to 13 mg/kg intraperitoneal weight) were used for this purpose, based on the De Carvalho master's dissertation [7] and the Silva doctoral thesis [1].

Subsequently, lungs and tracheas were collected. The upper portion of the trachea and the left lung were stored in a plastic bottle containing 10% formaldehyde (phosphate buffer, pH 7.4), such hemisections were dehydrated in increasing solutions of ethanol at 70, 95 and 100º GL, diaphonized in xylol and included in paraffin (conventional histological technique), according to the routine protocol of histological procedures [10]. They were reduced to a thickness of 5 µm and stained with hematoxylin and eosin (HE).

For analysis of variables of stress oxidative: malondialdehyde (MDA), sulfhydryl (SH) both in trachea and lung, catalase (CAT) in trachea, cytokines inflammatories: interleukin 1-β (IL-1β), tumoral necrosis factor α (TNF-α), both in the trachea. Were calculated the mean and standard deviation (SD) in each group. Furthermore, was made analyses descriptive of histology. The normal distributions analyses with the Test Shapiro-Wilk was applied and then was made the test Scott-Knott. The analyses were made comparing the CG with G24, G48 and G72. Was pre-determined the level of significance in 95% ($p<0,05$). The program used for statically analyses was the SPSS 20.0.

RESULTS

Histopathological changes induced by inhalation in the tissue of the trachea and lung of rats

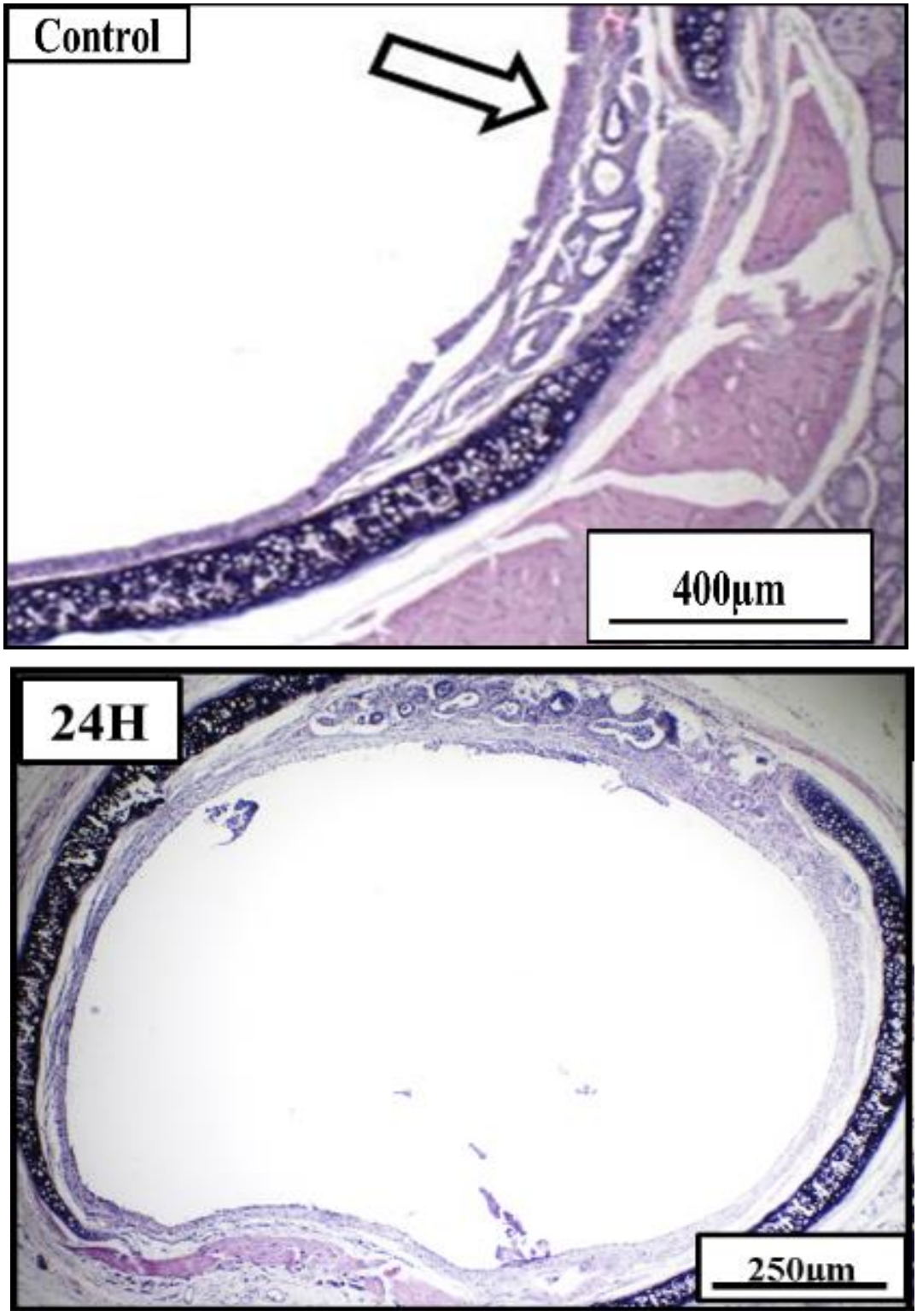
The main histopathological changes were observed in the tissue of the trachea and lung in all groups, mainly when compared to normal animals.

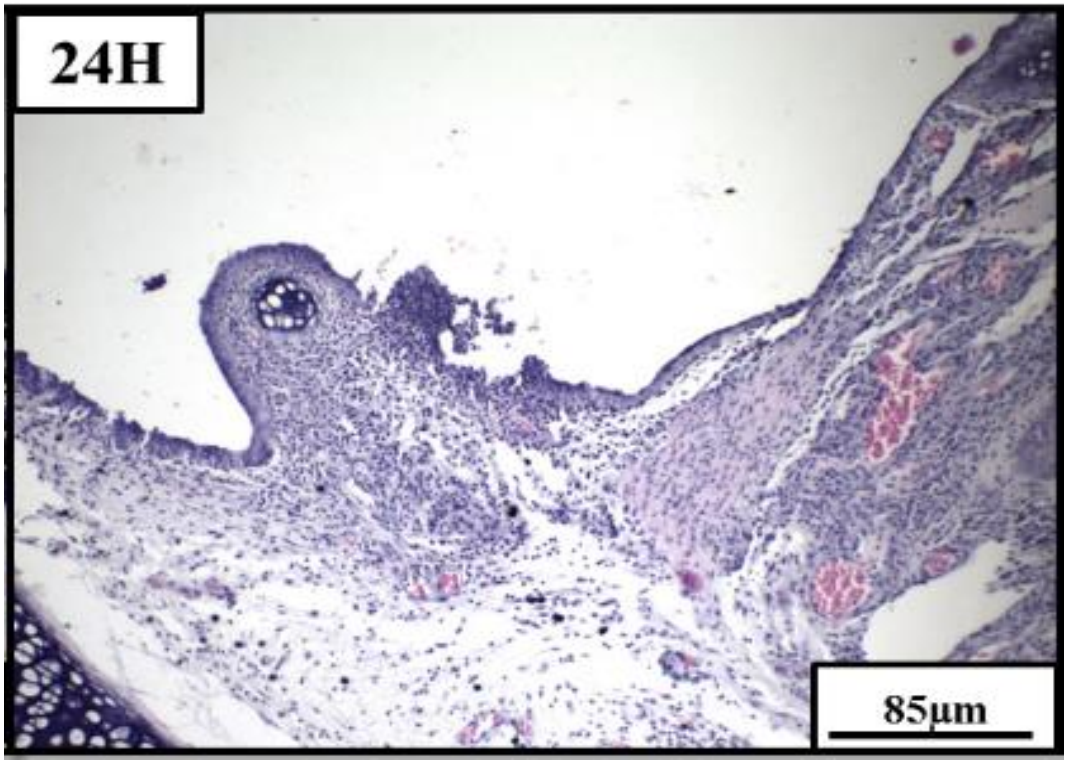
Trachea

In the control group (CG) shows that the trachea consists of four layers: the mucous (composed of a pseudostratified ciliated epithelium), submucous (dense connective tissue no modeled), cartilaginous layer (composed of hyaline cartilages) and the adventitia (that joins the trachea adjacent structures). They have ciliated cells in all extension of mucous layer of airways; mucous cells; have a basal membrane that consists of densely packed collagen fibers, own blade, layer submucous formatted by loose connective tissue (have the biggest vases).

In a group of 24 hours (G24) was observed sign of acute lesion, with presence of ulcerations and exulcations in epithelial tissue, a high hyperemia and presence of squamous metaplasia of epithelial tissue, as well as inflammatory infiltration composed of neutrophils and

lymphocytes. In G48, had areas of diapedesis, signal of modulation of inflammation, with presence of areas of chronification of the intense inflammatory response, with presence of neutrophils presence, eosinophils, too much hyperemia and interstitial edema. In G72, was observed destruction total of ciliated epithelium, as well as the remarkable reduction edema and repair of epithelial tissue.

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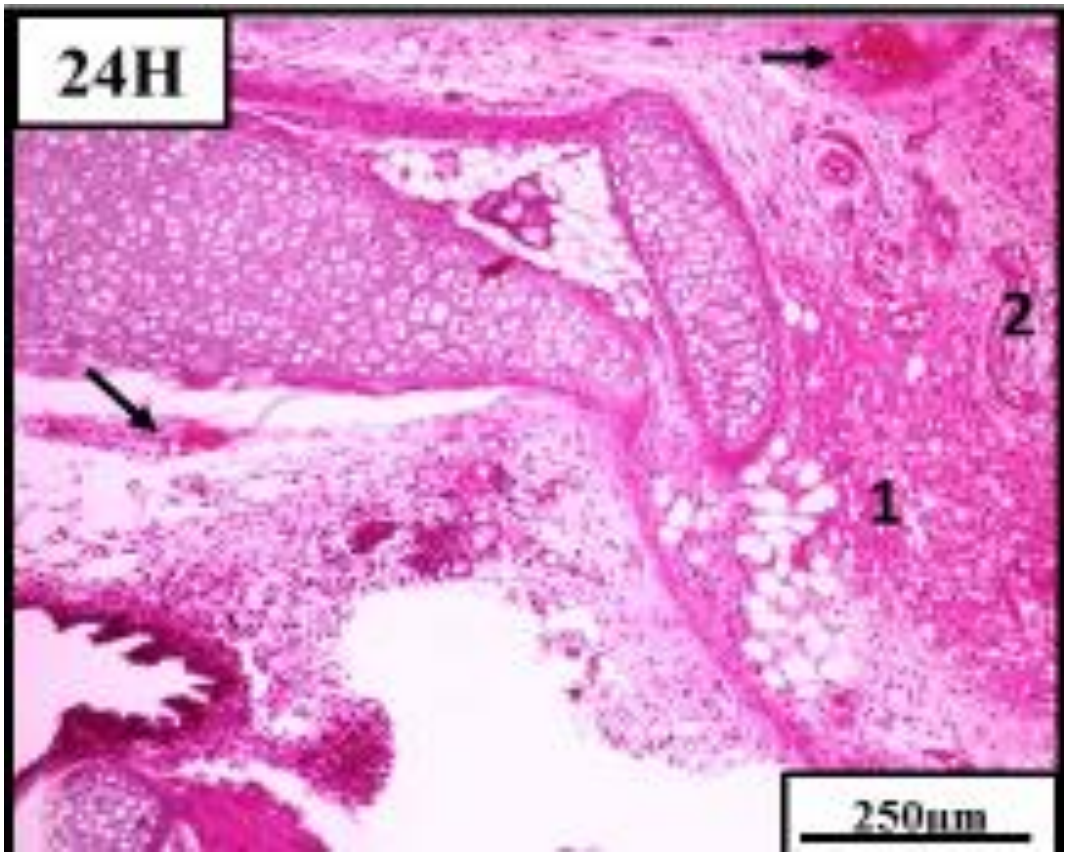
24H
2
1
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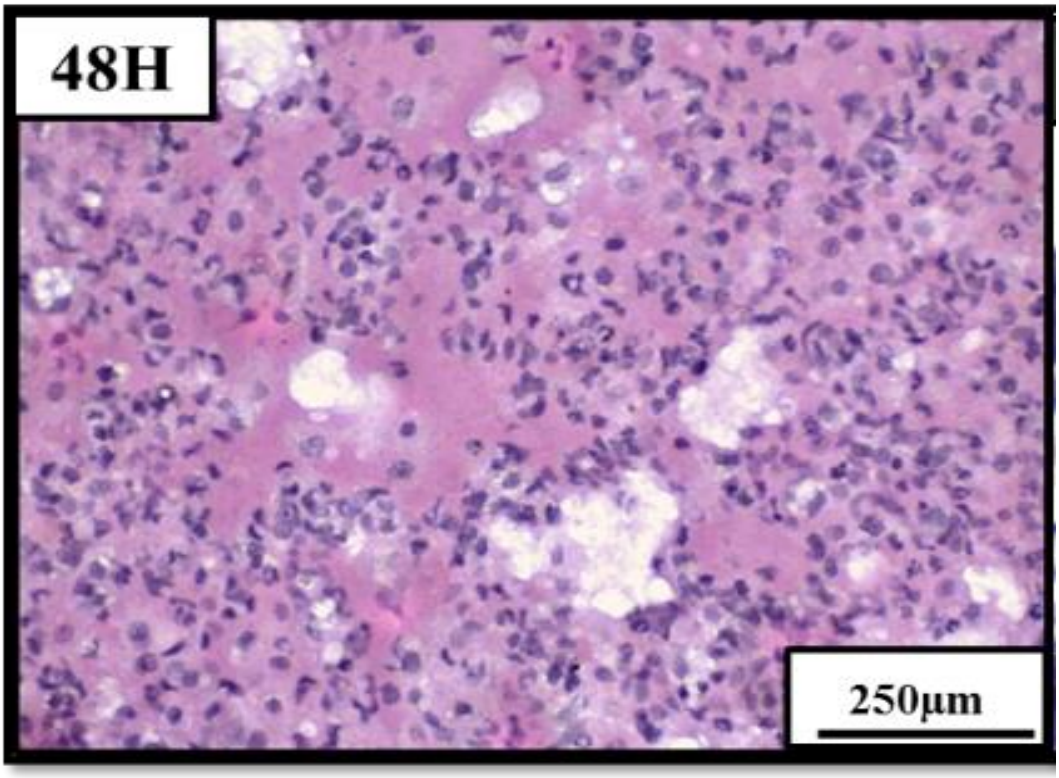
48H
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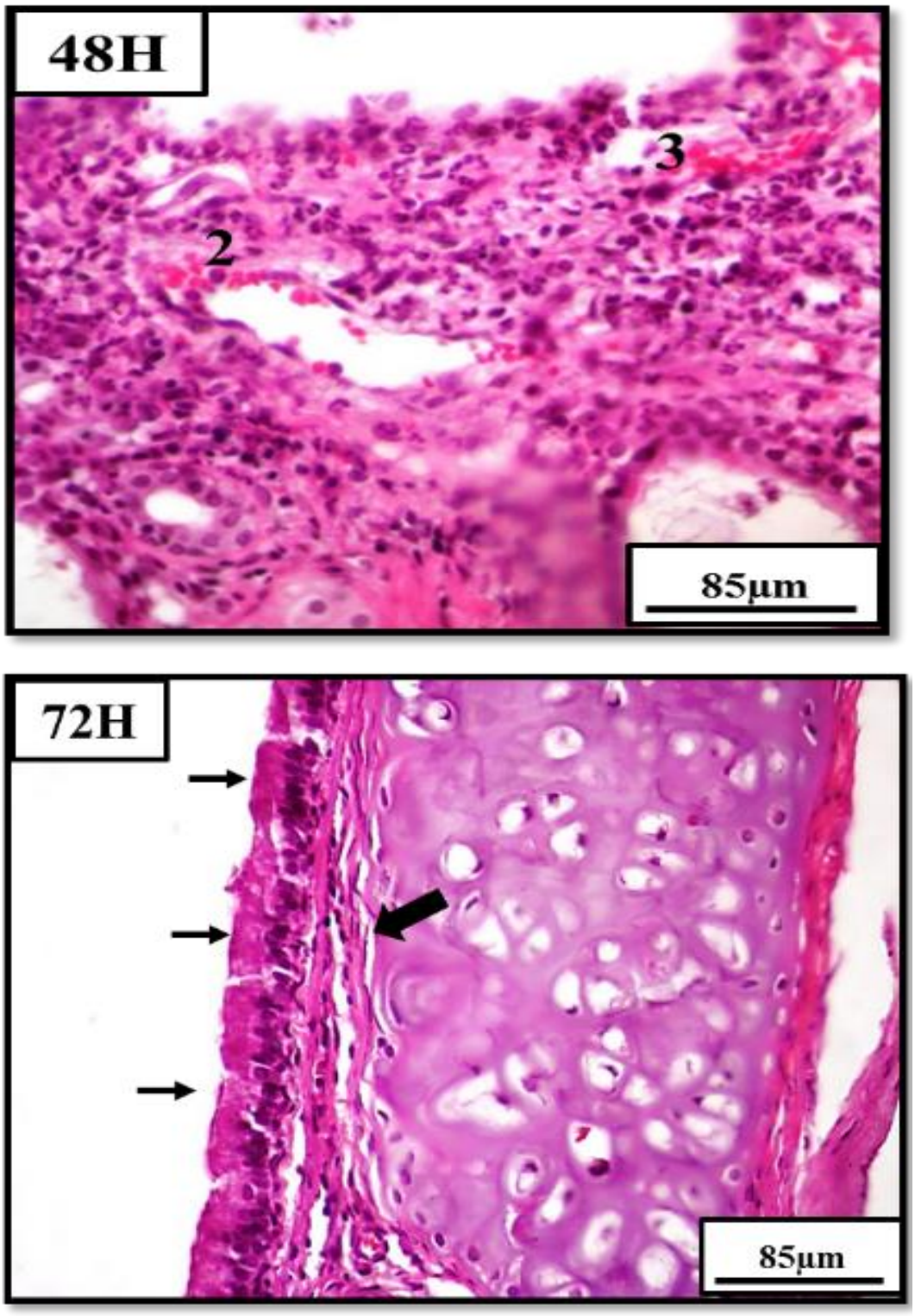
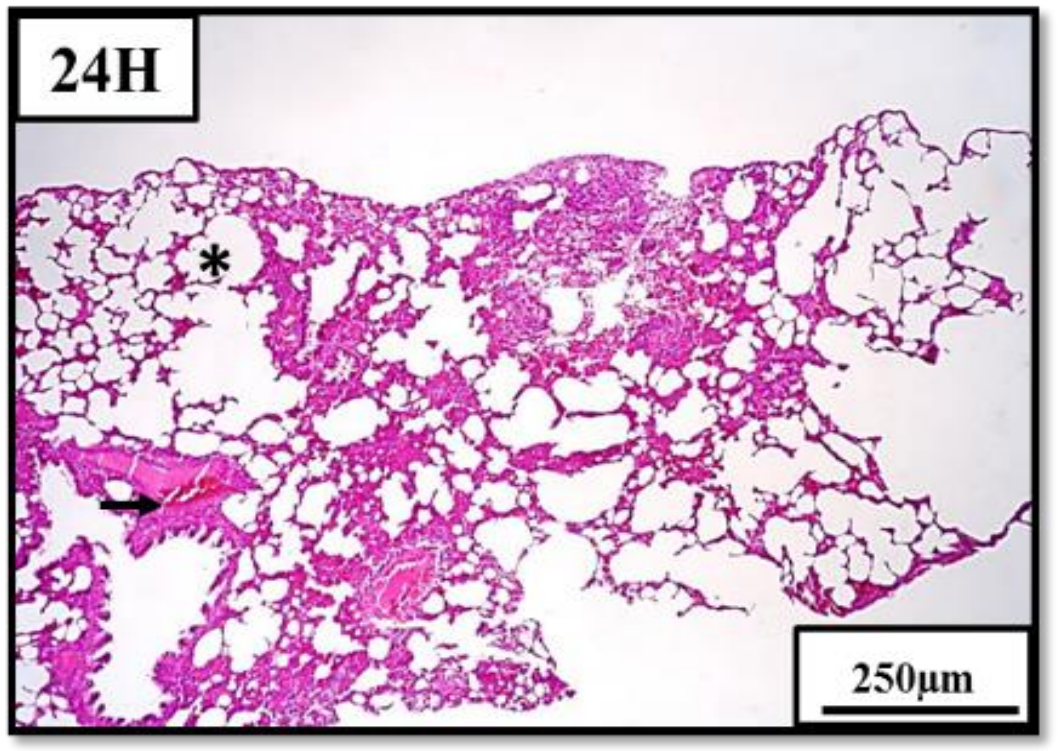


Figure A. Histological analysis of the trachea in time of 24, 48, 72 hours and CG colored Hematoxylin-Eosin (HE). Trachea in 24 h with interstitial edema and intense inflammatory infiltration rich in neutrophils in 1 and 2. Trachea in 48 h with diapedesis in 2 and hyperemia in 3. Trachea in time of 72 hours, thin arrows - destruction of ciliated epithelium; thick arrows - fibrosis onset. Control group show ciliated epithelium preserved.

Lung

In the control group (CG) shows the bronchioles have no layer cartilages neither glands. The terminals bronchioles are coated by simple cuboid epithelium containing Clara cells. Each alveolus contains a thin-walled polyhedral layer, confluent with the alveolar sacs, which are spaces surrounded by groups of alveoli. Finally, it presents an aerial hematobarrier composed of a thin layer of surfactant, a type I epithelial cells, a basal lamina and a capillary endothelial cell.

In a group of 24 hours was observed hyperemia, with areas of bleeding and evident emphysema, enlargement and destruction of alveolus walls. In 48 hours following with intense hyperemia, intense inflammatory response, signal of modulation of inflammation, with presence of neutrophils, eosinophils, lymphocytes, interstitial edema, enlargement and destruction of alveolus walls and evident emphysema. In time of 72 hours, alterations emphysematous and the alveolar architecture, besides areas of fibrosis.


24H
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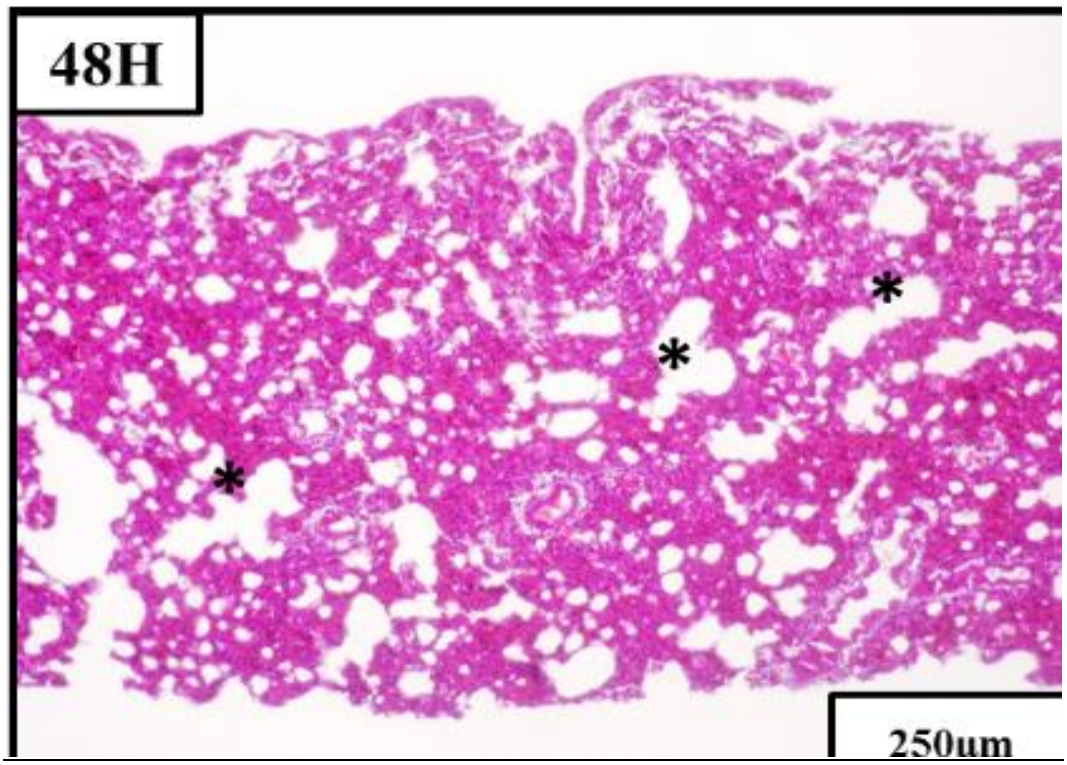
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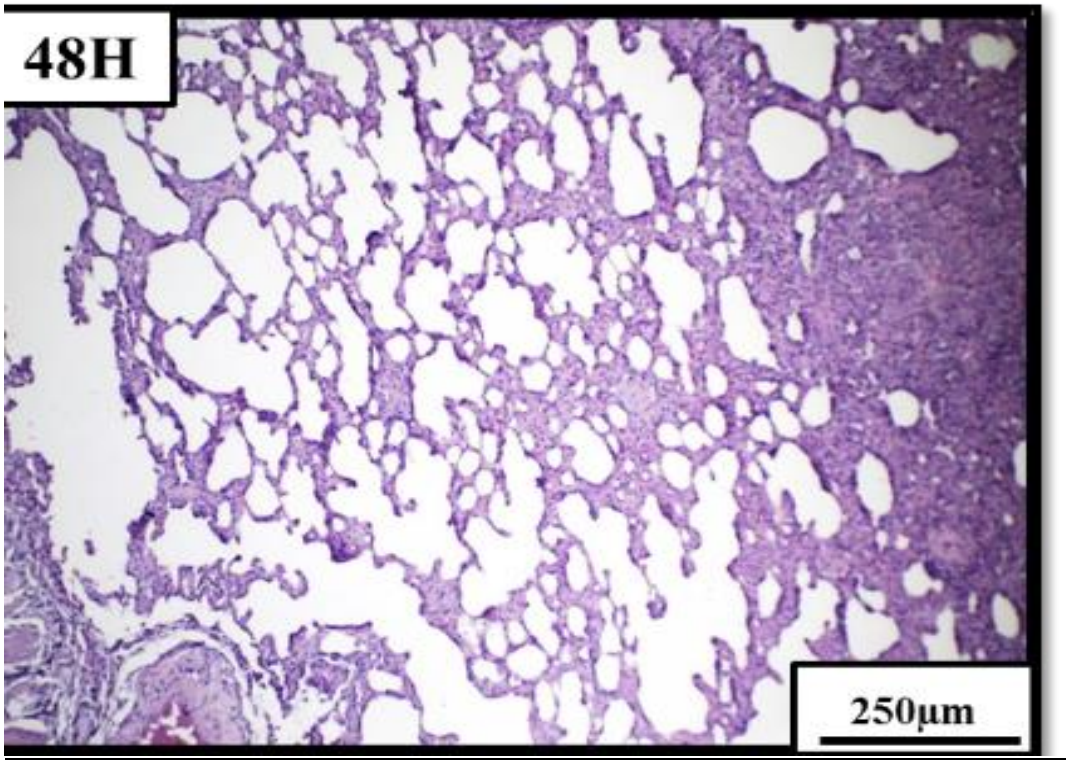
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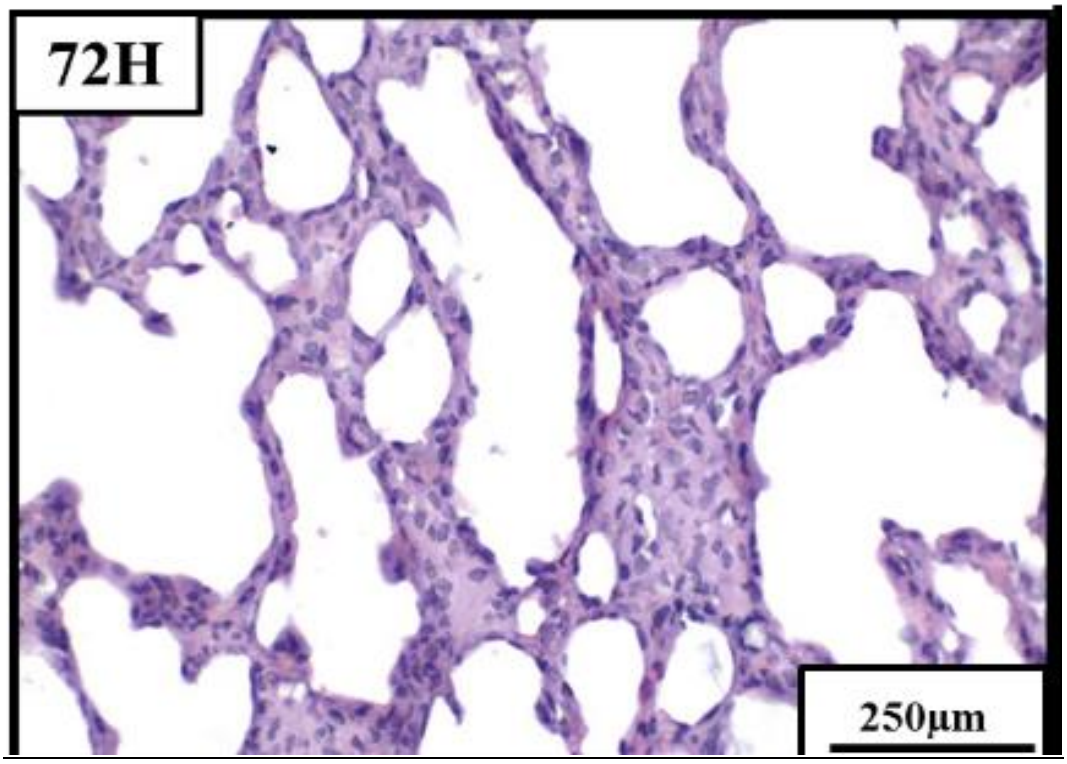
72H
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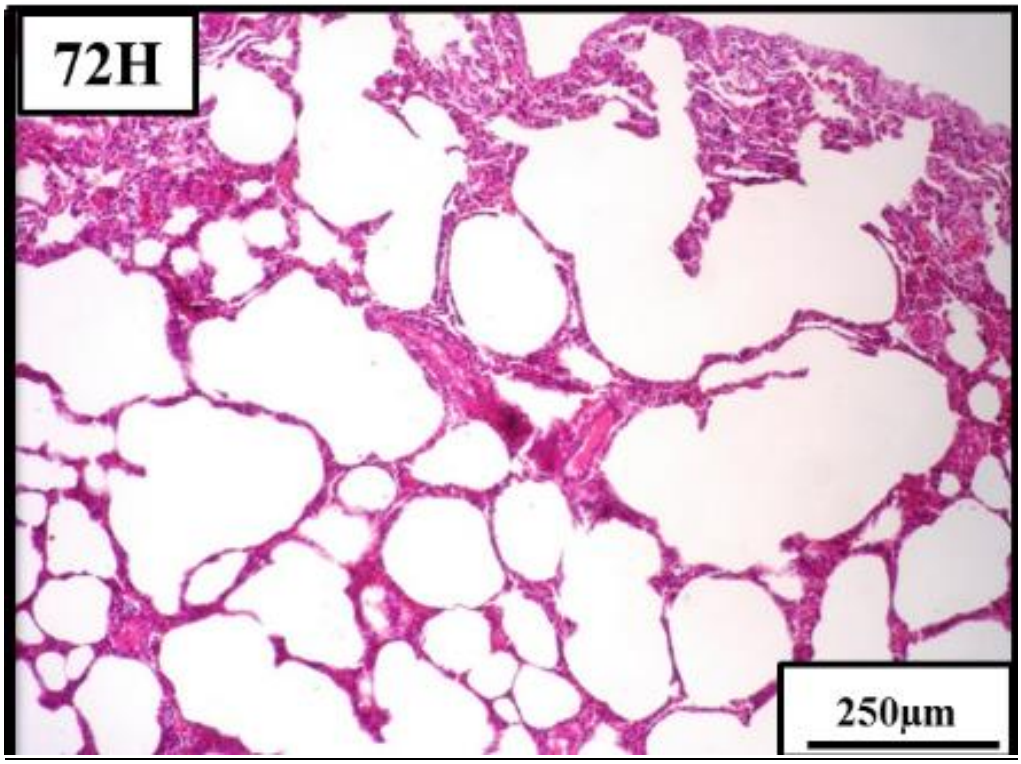

Figure B. Histological analysis of the lung in time of 24, 48, 72 hours and CG colored Hematoxylin-Eosin (HE). Lung in 24 h with interstitial edema and inflammatory infiltration (*) and arrow show hyperemia. Lung in 48 h with enlargement and destruction of alveolus walls and evident emphysema (*). Lung in time of 72 hours, thin arrows - alterations emphysematous and the alveolar architecture.

Action of oxidative stress in tissue of the trachea and lung of rats

There was a significant difference in lung tissue (p=0.00) relative to malondialdehyde (MDA) when compared to control group (CG) and all groups (G24, G48 and G72) (Fig. 3). There was a significant difference (p=0.01) relative to sulfidril (SH) in all groups (G24, G48 and G72) the trachea compared to CG (Fig. 4). However, in lung tissue, there was no significant difference (p=0.402) between all the groups (Fig. 5). There was a significant difference (p=0.02) of catalase (CAT) between group 24 hours compared to 48, 72 hours and CG in lung tissue (Fig. 6).

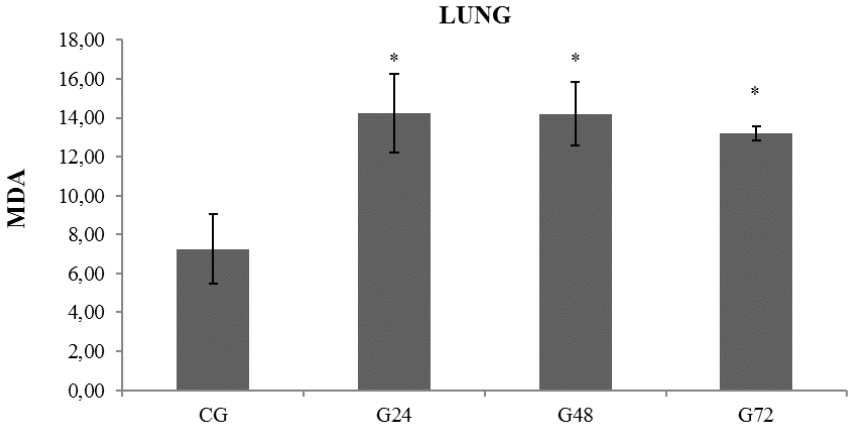

Figure 3. Effect of smoke induced injury in male rats at 24, 48, 72 h and CG post-smoke inhalation. Data obtained from tracheal tissue. Statistical differences between control group vs injury group: $^{*}p < 0.05$.

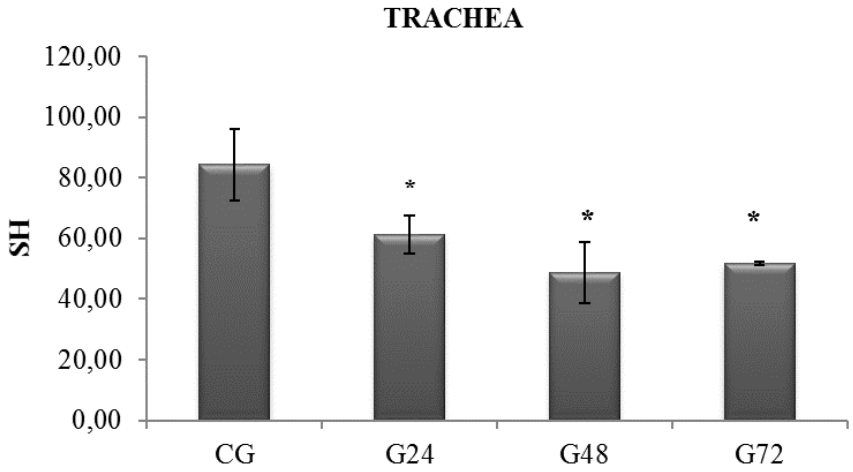

Figure 4. Effect of sulfidril (SH) in male rat post-smoke inhalation at 24, 48, 72 h and CG. Data obtained from tracheal tissue. Statistical differences between control group vs injury group: $^{*}p < 0.05$.

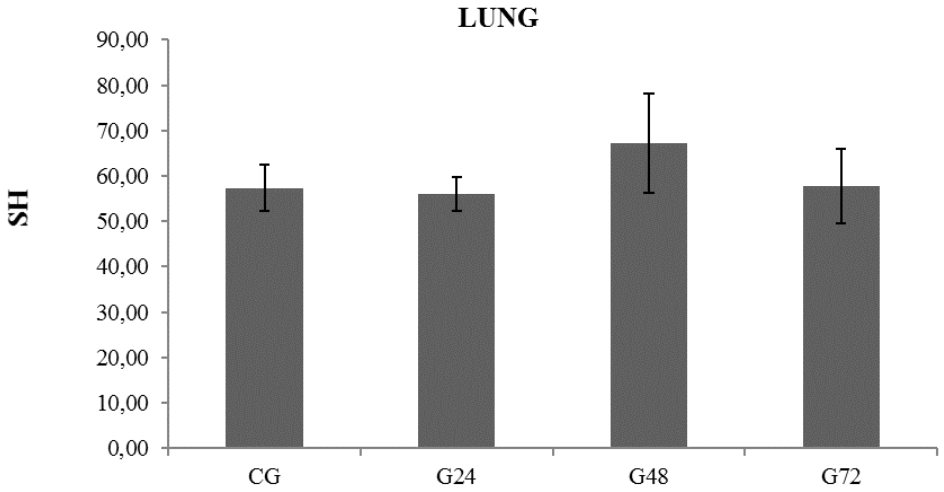

Figure 5. Lack of effect of sulfidril (SH) in male rat post-smoke inhalation at 24, 48, 72 h and CG. Data obtained from tracheal tissue. Statistical differences between control group vs injury group: $p > 0.05$.

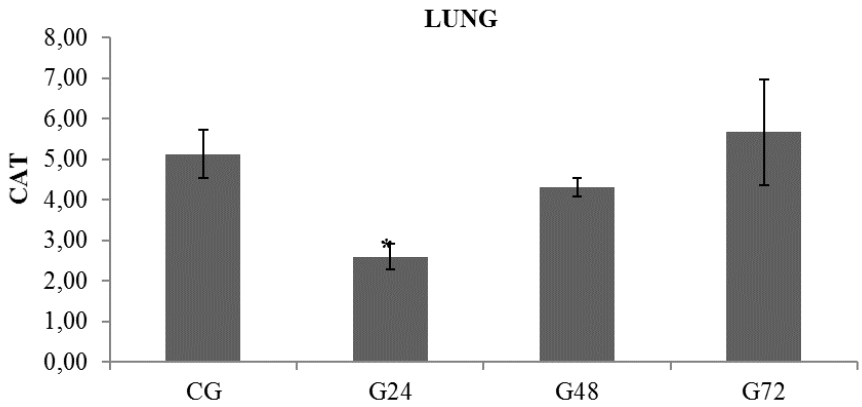

Figure 5. Effect of catalase (CAT) in male rat post-smoke inhalation at 24, 48, 72 h and CG. Data obtained from tracheal tissue. Statistical differences between control group vs injury group: $^{*}p < 0.05$.

Action of TNF-α and IL-1β in tissue of the trachea of rats

In relation to TNF-α there was difference significant (p=0.01) in G24 compared to CG when compared to CG (Fig. 6). Besides, the same occurred relative the IL-1β (p=0.00) in G24 compared to CG (Fig. 7).

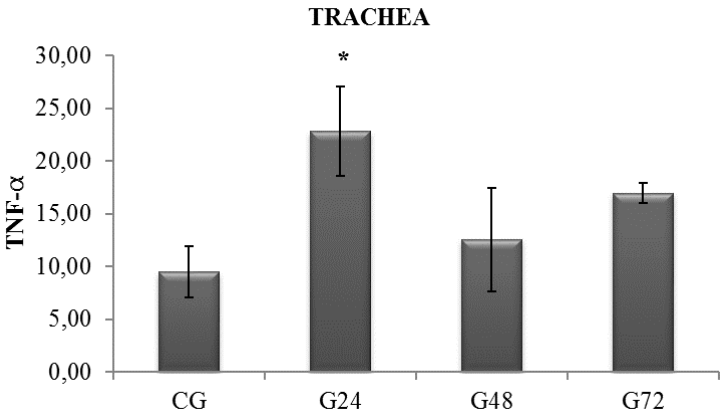

Figure 6. Effects of smoke inhalation on tumoral necrosis factor α (TNF-α) of rat post-injury at the 24, 48, 72 h and CG. Statistical differences between negative control group vs oxygen group: $^{*}p < 0.05$.

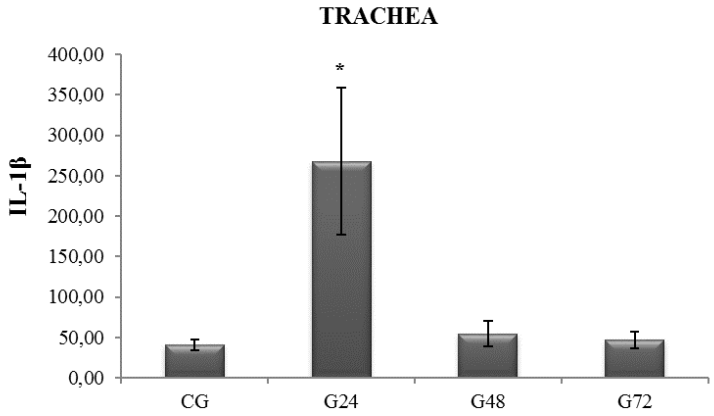

Figure 7. Effects of smoke inhalation on interleukin 1-β (IL-1β) of rat post-injury at the 24, 48, 72 h and CG. Statistical differences between negative control group vs oxygen group: $^{*}p < 0.05$.

DISCUSSION

Smoke inhalation release particles and toxic substances that cause the pathophysiologic changes in trachea and lung. Besides, the interaction of these substances in the trachea and lung parenchyma may trigger an inflammatory cascade reaction, that result in edema, congestion, small airway pulmonary, pulmonary hypoxia [12]. It was evidenced in the present study, that the exposure to cotton smoke during three periods of exposure (total of nine minutes each) was sufficient for generating an acute process inflammation with structural changes in tracheal and pulmonary, as found in previous studies of Matos [14] Silva [1] and Carvalho [13]. All groups demonstrated histological changes in the trachea and lung tissue. However, the major changes were in time of 48 hours in group of trachea and lung.

The inflammation process is due to the activation of pro-inflammatory cytokines through alveolar macrophages and direct or indirect oxidative stress. The tumor necrosis factor-α (TNF-α) is one of the cytokines responsible for the acute phase response to inflammatory stimuli, as well as the interleukin 1-B (IL-1β) acts in conjunction with TNF-α in natural immunity and inflammation. IL-1β acts primarily in the synthesis of acute phase plasma proteins, neutrophil production and platelets [15]. This increase corroborates the structural changes of the trachea present in G24.

The G24 of trachea showed squamous metaplasia, characterizing the changes of pseudostratified ciliated columnar epithelium to rocky stratified epithelium, that is more resistant to physical stress and aggression, but is less effective for function of airways. These findings were shown in book of Ross (2016) chapter 19 [16].

These changes cause inflammatory responses, soon zones of hyperemia begin to appear. It is caused by high blood circulation tracheobronchial, which up around ten times more for trachea, besides increasing six times to the left lung and four times to the right, as Enkhbaatar et. al (2003) found in his study [17]. Pulmonary tissue in G24 also has hyperemia and squamous metaplasia. Moreover, there is a presence of interstitial edema (presence of a lot of stroma with thickening of the interalveolar septum) due to inflammatory reaction with the release of proinflammatory cytokines such as TNF-α and IL-1β, and rupture of some alveoli which makes gas exchange difficult, as shown in a study of Matos et al (2017), Silva (2018) [1].

It is known that with the onset of gas combustion and the consequent inhalation of smoke, there is an increase in cellular metabolism. Associated with this, in this process of injury, hemoglobins (Hb), which have little affinity with oxygen molecules (forming oxyhemoglobins), bind more easily with carbon monoxide (CO) molecules, forming a complex called carboxyhemoglobin. CO has about 200 to 300 x more affinity with Hbs, resulting from the combustion process of burned materials [4]. This new bond induces the release of free radicals or reactive oxygen species (ROSs), which are highly reactive, unstable and can cause cell death [18]. Knowing that the half-life of CO is 4 to 6 hours; with oxygen therapy at 100%, it decreases to 6 to 75 minutes [5].

Due to this process of pairing the electrons of the last layer, there is the release of free radicals, such as hydroxyl (OH-), which, associated with neutrophil activation process, will damage the tissues more suddenly in the G24 [19]. This reaction directly infers the findings of the study, in which MDA - marker of oxidative stress - there was a significant difference at all times in the lung groups when compared to the control group.

Carvalho et al (2019) [13] found in their research, with the same model of lesion in rats, a significant increase in serum MDA levels, a fact that corroborates the results obtained in this research, that indicate smoke inhalation injury causes oxidative stress by increasing the MDA marker, when compared to normal group in trachea tissue mainly in the beginning injury [13, 21]. Moreover, these findings corroborate the systematic review of Carvalho [7], that the more acute the lesion, the greater the local blood circulation [1], consequently, the greater the release of inflammatory cytokines and oxidative stress.

The continuous production of free radicals during cells metabolic processes has led to the development of many antioxidant defense mechanisms, such as the endogenous enzyme catalase (CAT). Antioxidants are substances that, even at low concentrations, are able to delay or inhibit oxidation rates, as found in this study, that there was a sharp drop in serum catalase levels within 24 hours. However, in the 48 and 72 h groups there was an increase of these levels in an attempt to combat oxidative stress. Catalase helps reduce the effects of stress and lack of O_2 by forming complexes that attenuate free radical-producing reactions by converting hydrogen peroxide to H2O and O_2 [4, 18].

In addition, due to high blood circulation, areas of diapedesis begin to appear in a group of 48 hours of trachea and lung. This is because the immune system tries to eliminate any foreign body present by sending leukocytes from blood to connective tissue, and chronification of the intense inflammatory response, which presents much neutrophils presence, eosinophils, too much hyperemia and interstitial edema, that will more extensive tissue damage promoted by smoke and high inflammatory react. Supporting our findings, Silva (2018) Carvalho (2016) founded reaction of the granulation and thickening below the tracheal mucosa. Further, squamous metaplasia in the extension of the tracheal epithelium, originating stratified floor epithelium [1, 9].

According to the time of the lesion, there may be a decrease in the enzymatic activity of CAT and SH in trachea and lungs due to a reduction in pH below 4, but levels rise again between 4 and 8.5 as a consequence of hyperoxia, administered for 30 minutes at 100% FiO2, simulating rescue to the victim. As found in our studies, the pulmonary CAT and tracheal SH increased according to the groups studied (G24, G48, G72). However, an alteration in SH concentration in G48 of lung was shown, surpassing GC normality levels, with a decrease in G72. This finding is possibly due to the fact that there is greater neutrophilic activity and oxidative stress in G48 of the lung when compared to G24 and G72.

Our results showed characteristics of pulmonary emphysema, which are destruction and thickening of the alveolar wall and abnormal repair. Instead of this process, associated with the

time of lesion, the tissue will form areas of tissue fibrosis [19]. Added to this, it is known that the development of emphysema is not only correlated with the imbalance between protease activity and antiprotease, but also with the imbalance between oxidants and antioxidants, which, in turn, are related to inflammation in pulmonary pathology.

In our studies, sulfhydryl (SH), which is an antioxidant enzyme, showed no significant difference in lung. However, there was an increase in serum levels in G48, which exceeded the levels of GC normality. This result, possibly, may be related to the increase in the amount of catalase (CAT) in the lung, also an antioxidant, which converts free radicals, such as oxygen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2) [20, 21]. This action may be associated with the conversion of MDA into less reactive species. Thus, the antioxidant enzymes constitute the main mechanism of protection of the pulmonary parenchyma against lesions mediated by free radicals. Perhaps, this increase in SH in G48 occurred to try to reduce cellular metabolism, with consequent destruction of the alveolar wall, which will affect the integrity of collagen and elastic fibers. Similarly, to what occurred in this study, in the researches of Carvalho et al (2019) [13], the oxidative stress of MDA did not give significant, suggesting a positive action of the antioxidant activity (catalytic).

The process of tissue healing, which generates fibrosis in the lung, was confirmed in G72, which corroborates the initial stages of the chronic phase. These damages to the pulmonary parenchyma acutely exposed to smoke include moderate to intense interstitial inflammation, increased alveolar wall thickness, and destruction due to activation of neutrophils, which release proteolytic enzymes that destroy elastin in the pulmonary structure, as well as the release of superoxide radical, which directly damages the membrane of endothelial and interstitial cells. These findings coincide with those of Matos and Silva [14, 1].

The study may have presented a limiting factor related to the sample size. In addition, it is known that there are structural differences between small and medium sized animals when compared to humans, which is also a limiting factor that requires further studies.

CONCLUSION

Therefore, based on the data obtained, we can state that exposure to smoke inhalation is capable of inducing serious damage to the respiratory system, such as in tracheal and pulmonary structures, mainly by the action of inflammatory cells, such as neutrophils within 24 and 48 hours. In the tracheal tissue, these alterations are also related to the inflammatory activities of cytokines (TNF-α and IL-1β) present in the 24-hour period, which corroborates the action of oxidative stress, which has a higher level of pro-oxidant enzymes and lower levels of

antioxidants in the 24-hour period. Moreover, in lung tissue there is a greater alteration in G48, mainly structural, which is related to the action of oxidative stress.

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