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MENTHA ARVENSIS LEAF EXTRACT EFFECT ON REDUCING NEUTROPHIL CHEMOTAXIS IN PULMONARY TISSUE OF *RATTUS NORVEGICUS WISTAR*

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Abstract: **Purpose**: To evaluate the effect of mint leaf in reducing the neutrophil chemotaxis in lung tissue of *Rattus norvegicus wistar.* **Methods**: Twenty-eight Wistar rats were divided into four groups. In Group 1 (sham), only a laparotomy was performed. In Group 2 (positive control), the laparotomy was followed by upper mesenteric artery clamping and administration of beclomethasone via nebulization for ten minutes. In Group 3 (negative control) only the laparotomy and clamping were done. In Group 4 (mint), the laparotomy and clamping were followed by nebulization of mint leaf extract for ten minutes. All clamps were removed after forty-five minutes from their insertion and treatments, when performed, were instituted soon after. After ninety minutes of reperfusion, right lung base tissue samples were collected and properly stored from all rats. **Results:** In both Groups 2 and 4, there was reduction in inflammation in comparison to Group 3. Group 1 showed the lowest inflammatory cell count. Comparing average inflammatory cell counts of all groups to each other, there was statistical significance among all, except between groups 2 and 4. **Conclusion**: These results show that mint leaf extract is able to, trough nebulization treatment, significantly reduce the neutrophil chemotaxis in pulmonary tissue of *Rattus norvegicus wistar* subjected to induced acute lung injury.
Keywords: Mentha, Inflammation, Neutrophils, Chemotaxis

INTRODUCTION

Acute lung injury (ALI) is characterized by an inflammatory process, mainly of neutrophilic origin, which leads to diffuse alveolar lesion, pulmonary edema and surfactant dysfunction (Mokra and Kosutova 2015, Bernard et al. 2011). It can be caused by several conditions, such as sepsis, pneumonia, trauma, acute pancreatitis, aspiration of

gastric contents, among others. The disease manifests early with tachypnea, tachycardia and respiratory alkalosis. Then, there is formation of pulmonary edema, which leads to a ventilation-perfusion mismatch and reduced pulmonary compliance, culminating in severe hypoxia (Mokra and Kosutova 2015, Dushianthan et al. 2011).

The inflammatory cascade triggered by lung injury involves the activation of cells in the immune system and the release of chemical mediators. Neutrophils, once activated, undergo a capture and rolling process mediated by the interaction between L-, E- and P-selectins. The rolling process is followed by activation of cytokine-dependent integrins that ultimately lead to adhesion to the capillary endothelium and the migration of neutrophils to interstitial and alveolar spaces. Thus, after migration, neutrophils produce cytokines, granular proteins (e.g., proteolytic enzymes and cationic peptides) and reactive oxygen species that leads to tissue damage, playing a crucial role as signaling molecules that initiate, amplify and perpetuate the inflammatory response, both local and systemic (Mokra and Kosutova 2015, Ley et al. 2007, Cross and Matthay 2011, Grommes and Soehnlein 2010). Evidence of this is the direct correlation between neutrophil levels with severity and the risk of progressing to an unfavorable outcome (Steinberg et al. 1994). Although the role of neutrophils in these changes is crucial, pro-inflammatory mediators are also produced by other cells, such as alveolar macrophages (Mokra and Kosutova 2015).

Increased levels of pro-inflammatory cytokines (e.g., IL [interleukin] -1, Tumor Necrosis Factor [TNF-a], IL-6 and IL-8) as well as decreased anti-inflammatory drugs (e.g., IL-10 and IL-13) can be detected in plasma or bronchoalveolar lavage in response to immune cell recruitment or cell death (Ley et al. 2007). Thus, ALI tissue damage is characterized by increased alveolar endothelial and epithelial permeability, responsible for pulmonary edema, surfactant dysfunction and hematosis disorders (Grommes and Soehnlein 2010). ALI can be generated experimentally by ischemia in extrapulmonary sites, such as by clamping the superior mesenteric artery (SMA) for a period and successive reestablishment of the perfusion. The lung injury in this model depends on the direct action of polymorphonuclears (Koike et al. 1995).

Thus, the use of agents with anti-inflammatory activity can be a reasonable alternative in the management of ALI. Among natural products with therapeutic properties, vegetables have traditionally been used to cure various infectious and inflammatory conditions, including, among others, gastrointestinal, pulmonary, dermatological and systemic diseases (McKay and Blumberg 2006, Baliga and Rao 2010, Kalemba and Kunicka 2003). One of the most notable examples of this type of therapy is the use of mint to treat disorders related to the gastrointestinal tract (e.g., dyspepsia, enteritis, flatulence, gastritis, intestinal colic and bile duct spasms) and lung conditions, acting as an antitussive, decongestant nasal and ally in the treatment of asthma. There is also evidence of the protective action of *Mentha arvensis* against damage caused by radiation in rats, probably due to its antioxidant, antimutagenic and anti-inflammatory effects (McKay and Blumberg 2006, Baliga and Rao 2010, Juergens et al. 1998). In addition, it was shown that the previous treatment of cells with *Mentha arvensis* extract modulated the immune response by decreasing JNK expression and p38 MAPK phosphorylation and increasing ERK-1/2 MAPK phosphorylation, both the former being involved in the induction and production of pro-inflammatory cytokines

(IL-1, IL-6, TNF-a) and the latter acting as a negative regulator of this process (Yadav and Chandra 2017).

Therefore, based on the inflammatory modulating properties of the *Mentha arvensis* and the idea that the activation of inflammatory cells is the key to the progression of ALI, the present study aims to evaluate the anti-inflammatory action of the ethanolic extract of *Mentha arvensis* on induced ALI by an ischemia-reperfusion model in extrapulmonary site in rats.

MATERIALS AND METHODS

The project was approved by the Ethics Committee on the Use of Animals (protocol Nº 249/16). All the procedures were performed in accordance with the rules issued by the National Council for the Control of Animal Experimentation and with the precepts of Law N° 11.794. For this purpose, female (more available than male in the period) Wistar specimens (*Rattus norvegicus wistar*), weighing 200 to 250g, were supplied by the Central Animal Bioterium of the Agricultural Sciences Center of the Federal University of Piauí (UFPI). The rats were kept in polypropylene cages (up to 5 per cage), under controlled temperature and humidity conditions (23 - 25 °C and 40%, respectively) and a 12h light-dark cycle, starting at 6 am. The animals had free access to food and water.

Preparation of the extract and nebulization solution

Mint leaves (*Mentha arvensis*) were collected in the center of medicinal plants at UFPI, which were placed in a drying oven to remove humidity at a temperature of 45º to 50ºC, for 24 hours, and later submitted to the grinding process, in an industrial blender (METVISA – 26FEV14), at UFPI physiology laboratory.

After grinding, absolute ethyl alcohol was added in the proportion of 1:3 to obtain the oil, which was manually stirred for five minutes every two hours for 12 hours and subsequently filtered through a simple glass funnel and cotton funnel for three consecutive times. The ethanolic extract was concentrated on a rotary evaporator (IKA RV-10) under reduced pressure and controlled temperature (50 ° C - 55 ° C), until reached 50% *Mentha arvensis* absolute oil concentration. The resulting material was kept in a refrigerator throughout the development.

On the days of the experiments, the nebulization solution was prepared. 100ml of water were placed in a kitasato flask supported on a heated plate and, after starting boiling, the heat supply was interrupted. Then the mint leaf oil (50%) was added to the recipient until it reached a concentration of 5% (5g/100ml, 2,5g of absolute oil per 100ml) and waited until the solution reached room temperature. The extract concentration was determined so that each animal, at the end of the 10 minutes nebulization period, received about 400µL/kg of *Mentha arvensis* absolute oil (Sharma et al. 2018). It is worth mentioning that polysorbate was added to the oil for proper infusion and subsequent nebulization.

Induction of acute lung injury

For the induction of acute lung injury, was adopted an ischemia/reperfusion method in the SMA. One of the reasons for this choice was the fact that this method reproduces a clinical phenomenon already known in humans, which is the development of lung injury after an episode of intestinal or peripheral ischemia followed by reperfusion. In addition, it leads to the rapid development of polymorphonuclear lung infiltration after reperfusion is established (Grommes and Soehnlein 2010, Matute-Bello et al. 2008), facilitating the proposed analysis, described later.

The animals of the experiment were anesthetized with ketamine (50mg / kg) and xylazine (10mg/kg), administered intramuscularly. If needed, half an anesthetic dose was administered for maintenance during the surgical procedure.

Then, after median laparotomy and evisceration, intestinal ischemia was induced by clamping the SMA for 45 minutes, which was performed with microsurgical vascular clamps (Figure 1A). During the period of 45 minutes of ischemia, the abdominal incision was covered with a transparent plastic to minimize loss of fluid and heat (Figure 1B).

After the ischemia time, the vascular clamp was removed and, thus, the period of intestinal reperfusion started, which lasted 90 minutes. The animals allocated in groups to be treated received the respective solution via nebulization for 10 minutes right at the beginning of the reperfusion period (Figure 1C).

At the end of the reperfusion period, the animals were euthanized with ketamine overdose (150mg / kg) and xylazine (30mg / kg) and lung samples were collected for histological analysis. For this, the lungs were removed and the left lower pulmonary lobe was separated and submitted to the fixation process, remaining in 10% formaldehyde for 48 hours.

Figure 1: A – Clamping of the superior mesenteric artery with microsurgical vascular clamp after median laparotomy and evisceration. B – Cover of the abdominal incision with a transparent plastic. C – Animal receiving nebulization soon after reperfusion starts

Study design

Twenty-eight animals were used, randomly divided into four experimental groups of 7 animals each, the following being:

Group 1: Sham (only laparotomy performed)

Group 2: Positive Control (intestinal ischemia and reperfusion + nebulization with beclomethasone dipropionate 400 µg / ml)

Group 3: Negative Control (intestinal ischemia and reperfusion + 0,9% saline nebulization)

Group 4: Mint (intestinal ischemia and reperfusion + nebulization with mint leaf 5% oil solution)

Preparation for histological analysis

After fixation in 10% formaldehyde, the lung tissue was submitted to the dehydration process, remaining 24 hours in 70% alcohol and another 24 hours in 96% alcohol. Subsequently, it was immersed in absolute alcohol (100%) during the 8-hour period and then diaphanized in xylol and embedded in paraffin. Finally, the material was processed in a microtome, with 3µm thick histological sections, followed by staining procedures using the hematoxylin-eosin (HE) technique and preparation of the histological slides for further analysis.

Histological analysis

For this, an optical microscope of the Leica brand, model DM 1000, was used, coupled to a Leica DFC280 camera, using the Leica DFC Twain software, with the acquisition of photos at 400X magnification (Figure 2). To avoid analysis of isolated local inflammation, for each animal, four images of different randomly chosen fields were obtained to

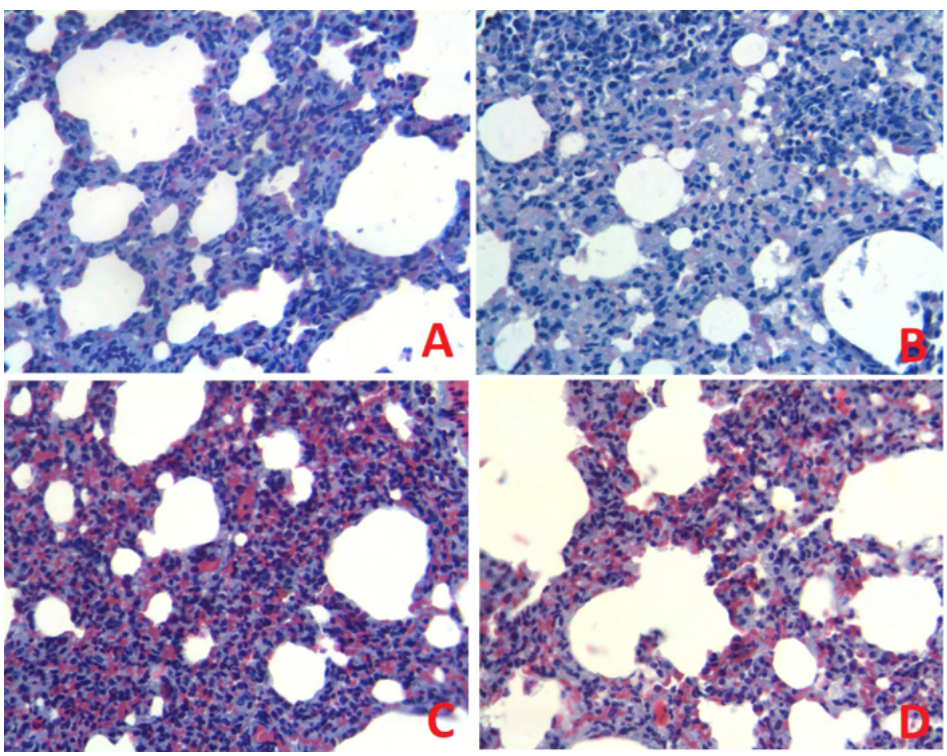


Figure 2: Photos in 400x magnification of slides prepared using materials collected from one animal of each group. A – Group 1 (Sham); B – Group 2 (Positive Control); C – Group 3 – (Negative Control); D – Group 4 – (Mint);

count the number of inflammatory cells (predominantly neutrophils).

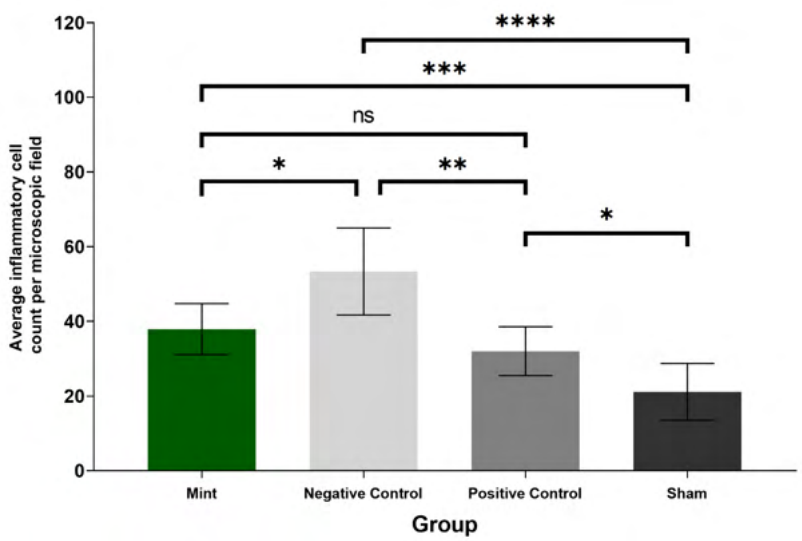
Compilation of results and statistical analysis

Once the data were collected, they were compiled via a Microsoft Office Excel spreadsheet and later analyzed statistically using the GraphPad Prism 8.0 software. First, normality tests were performed to determine whether cell count values followed a parametric distribution (Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov). Then, F tests were performed with all possible combinations of 2 groups to determine the need, or not, to apply Welch correction in t tests to be performed. Once the characteristics of the distributions were verified (isolated and comparatively), the appropriate statistical significance test was continued. It is worth mentioning that the value of "n" used in the test was 7, considering that, despite the use of 28 fields to perform the mean, the total number of experimental animals per group is 7. The results were expressed as the mean ± standard deviation (SD). Values of $p < 0.05$ were considered significant.

RESULTS

All groups (1, 2, 3 and 4) showed normal distributions of the 28 cell counts (4 fields per animal). In addition, when compared using the F test, the variances did not show any statistically significant difference. Therefore, we opted for the unpaired t test as evidence for the difference between the averages of cell count.

The average values of inflammatory cell count and respective standard deviations, as well as the presence of statistical relevance in the comparison between each of the 6 possible pairs, are compiled in Figure 3.



ns - $p > 0.05$

* - $p \leq 0.05$

** - $p \leq 0.01$

*** - $p \leq 0.001$

**** - $p \leq 0.0001$

Figure 3: Average of inflammatory cell count per microscopic field ± standard deviation for each group.

Analyzing the controls, it was observed that the lung tissue samples from Group 3 (negative control) had an average of inflammatory cellularity greater than that from Group 2 (positive control). In addition, among controls, Group 1 (sham), showed the lowest inflammatory cell count. All differences between controls were statistically significant ($p < 0.05$).

When comparing Group 4 (mint) with controls, there was a reduction in the number of average inflammatory cell count in comparison to group 3 (negative control) and the opposite in comparison to Group 1 (sham), both relations with statistic relevance ($p < 0.05$). The average inflammatory cell count was higher than in Group 2 (positive control), but without statistical significance ($p > 0.05$).

DISCUSSION

Mint leaves components have been found to express various effects on different human pulmonary diseases or symptoms. For example, the action of some components has been linked to the reduction of nasal sensation of airflow (Eccles et al. 1988) and inhibited production of arachidonic acid metabolites leukotriene 4 and prostaglandin E2 in monocytes from asthmatic patients (Juergens et al. 1998). Analyses of mint extracts or components in ischemia/reperfusion settings have been restricted to experimental animal models.

Acute lung injury is related and can be caused by the onset of mesenteric ischemia followed by reperfusion (Breithaupt-Faloppa et al. 2012, Cavriani et al. 2005). In this situation, a stimulus to oxidative aggression and endothelial and epithelial damage participates, in a big way, in an intense neutrophilic inflammatory response (Grommes and Soehnlein 2010, Ferro et al. 2010). Thus, the counting of this type of cells in the lung tissue is one of the parameters to measure inflammatory activity, in this study and in experiments that evaluate an ALI caused by obstruction of the mesenteric arterial flow (Thais et al. 2018, Rocha et al. 2014).

In the initial analysis of the results, it was observed that Negative Control (group 3) and Sham (group 1) had the highest and lowest amounts of inflammatory cells, respectively ($p < 0.0001$). This outcome was expected, given that the group 3 evolved with the processes resulting from mesenteric ischemia / reperfusion and was not treated and the group 1 did not receive the superior mesenteric artery clamping. It is important to note that the Sham group provides a baseline pattern for the level of inflammation caused by laparotomy, present in all groups, with significantly lower average inflammatory cell count compared to the others.

It was also observed that the positive control group had the lowest neutrophil count, compared to the negative control group ($p = 0.002$). This demonstrates the expected effect of beclomethasone dipropionate, a glucocorticoid with a known pulmonary anti-inflammatory effect (De Benedictis et al. 2015), related to the inhibition of phospholipase A2, blocking the formation of eicosanoids, which are derived from arachidonic acid, and suppressing NF-kB, reducing the expression of pro-inflammatory genes (Chassot et al. 2015).

In the group that received nebulization with *Mentha arvensis* extract (Mint), a reduction in the number of inflammatory cells was observed in relation to the negative control group ($p = 0.0132$). These data demonstrate that the anti-inflammatory effects performed by the components of this extract, already known in other study models (Shin 2003, Zaidi et al. 2012), possibly extend to situations of acute lung injury. However, further studies

are needed to elucidate which molecules and mechanisms are involved in this process and which active components have the greatest effect.

In addition, there was no statistically significant difference between the group that received nebulization with *Mentha arvensis* extract and the positive control group ($p = 0.1255$). The observed outcome may be related to the relative low intensity of inflammation promoted by the ALI assessment models preceded by extrapulmonary ischemia / reperfusion, a known disadvantage of this method (Matute-Bello et al. 2008).

The detailed study of the mechanisms of action of *Mentha arvensis* is still the subject of research. The role of Mentha arvensis in reducing MAPKs activation was described in alveolar macrophages (Yadav and Chandra 2017), what could be related to the effect observed in this study. This pathway`s activation leads to expression of pro-inflammatory genes and cytokines, such as IL-1, IL-6 and TNF-α (Chiu and Lin 2008) and phosphorylation of protein kinase B - PKB (Cuadrado and Nebreda 2010). In fact, there is evidence to support the role of this mechanism in impairing neutrophil chemotaxis, an effect directly related to this study's outcome - inflammatory cell count (Heit et al. 2008a, Heit et al. 2008b, Xu et al. 2013, Zu et al. 1998). Furthermore, elevated neutrophil count in pulmonary tissue of rats subject to induced acute lung injury has been associated with increased lung myeloperoxidase (MPO) mRNA and protein levels and IL-1 mRNA levels (Ahmad et al. 2019)

Despite being preliminary, studies have demonstrated the potential anti-inflammatory effect of active components, such as rosmarinic acid (Rocha et al. 2014). Moreover, its action in the inhibition of the NLRP3 inflammasome was observed by experimental studies (Wei et al. 2018, Yao et al. 2019). This action is particularly interesting, considering that, recently, the development of acute lung injury following ischemia/reperfusion in rats was related to this pathway's activation and subsequent IL-1B production (Ito et al. 2020).

Our study has some limitations. Since neutrophil presence in lung tissue was studied, it would be interesting to measure the concentration of myeloperoxidase and neutrophil elastase in the bronchoalveolar lavage fluid and inflammatory cytokines in plasma, as well as measure the inflammatory genes expression by molecular methods. It was not made due to financial limitations.

CONCLUSION

In this study, it was determined that mint leaf extract is able to, through nebulization treatment, significantly reduce the neutrophil chemotaxis in pulmonary tissue of *Rattus norvegicus wistar* subjected to induced acute lung injury.

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