Evaluation of inflammatory and oxidative profile in patients with recurrent wheezing

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\textbf{ABSTRACT}

Wheezing is a clinical expression of numerous respiratory-related conditions. Although asthma is the leading cause of wheezing during childhood. The present study aims to evaluate the inflammatory and oxidative profile of pediatric patients with recurrent wheezing. Seventy-eight volunteers were divided into three groups according to their age (≤ 36, 36 to 72 and ≥ 73 months). Blood was collected for hematological evaluation, serum detections of total immunoglobulin E (IgE), and C-reactive protein (PCRus). The oxidative profile was evaluated by total antioxidant capacity (FRAP), malondialdehyde (MDA) and carbonylated protein markers. There was no significant difference in the IgE and PCRus levels among the three groups evaluated. However, a significant positive correlation was observed for PCRus with total leukocyte and with neutrophils for the group of patients ≥ 73 months of age. The intermediate age group presented significantly reduced FRAP values in the serum, while significant values of oxidative damage markers were observed in the group of patients ≥ 73 months of age. When determining the correlation between inflammatory and oxidative markers, only the ≥ 73 months group showed significant. The group ≥ 73 months stands out with significant alterations of the oxidative stress markers and their correlations with the inflammatory profile.

\textbf{Keywords}: Wheezing; inflammation; oxidative stress; asthma
1 INTRODUCTION

Wheeze are multifactorial symptoms that indicate partial airway obstruction and relate to early-onset asthma in young children (Ducharme et al., 2014). Wheezing among young children is classified into three phenotypes: Transient Early Wheezing, Late-onset Wheezing, and Wheezing. Children who wheeze in the first three years of life but grow out of wheezing by age six years exhibit Transient Early Wheezing. Children who do not wheeze in early life but who present persistent wheezing at age six have Late-onset wheezing. Finally, children who wheeze in early life and continue to the age of six have general Wheezing (Ducharme et al., 1995). A family history of allergies and other atopic illnesses are risk factors for the development of wheezing episodes and persistent asthma (Amat et al., 2011, Bozaykut et al., 2013). With a complex etiology, asthma may result from the interaction between genetic and environmental factors (Lasso-Pirot et al., 2015), like virus-related respiratory infections (Ferriani et al., 2004), passive smoking (Lannerö et al., 2006), and psychosocial factors (Klinnert et al., 2013).

Recurrent wheezing results in an inflammatory imbalance related to early allergic sensitization to different antigens, with IgE production and participation of mast cells, eosinophils, lymphocytes and activated macrophages in the inflammatory process (Sherrill et al., 1999; Wright, 2002). These cells also act in the pathogenesis of asthma, through the IL-5 (Wang et al., 2016) and IL-9 cytokines (Hauber & Hamid, 2005), IgE (Froidure et al., 2016) and reactive oxygen and nitrogen species (ROS, RNS) mediators (Kleniewska & Pawliczak, 2017). Children predisposed to persistent wheezing present high levels of total IgE in the first months of life, preceding allergic sensitization. In asthmatic children, high titers of IgE increase the risk of rhinovirus-triggered wheezing episodes (Soto-Quiros et al., 2012). The C-reactive protein (PCR) can be present at high levels, although in asthmatic adults there is no significant difference in PCR between atopic and non-atopic patients (Fujita et al., 2007).
Moreover, oxidative stress is a component of asthma development and persistence (Jesenak et al., 2017). The production of ROS/RNS can generate lipid peroxidation as well as damage to proteins and DNA, with functional changes (Kurutas, 2016; Kleniewska & Pawliczak, 2017). Also, it can enhance hyperactivity and contraction of airway smooth muscle, increase vascular permeability with airway edema, and impair response to bronchodilators through the synthesis of cytokines, which contributes to asthma endurance (Jesenak et al., 2017).

Wheezing and coughing are the clinical expressions of numerous respiratory-related conditions. Although asthma is the leading cause of wheezing during childhood (Wright, 2002), most cases present spontaneous remission. This study aimed to determine the inflammatory and oxidative profile of children with recurrent wheezing, among different ages, evaluated at the Specialized Care Center of Viçosa, Minas Gerais, Brazil.

2 METHODOLOGY

2.1 Study design

Seventy-eight pediatric patients from the Pulmonology Department of the Specialized Care Center (Viçosa, Minas Gerais) agreed to participate voluntarily in the study. The patients were selected based on the following criteria: (i) recurrent wheezing patients of both sexes; (ii) who were in attendance in the mentioned center and were between 0 and 18 years old; (iii) residing in Viçosa or the surrounding region. The study excluded patients who had other associated diseases like heart disease, cystic fibrosis, gastroesophageal reflux disease, pneumonia, pulmonary tuberculosis, bronchopulmonary dysplasia, cerebral palsy, pulmonary congenital malformations, immunodeficiencies and postinfectious bronchiolitis obliterans.
Participants were divided into three groups according to their age: ≤ 36 months (lactents), between 37 to 72 months (pre-school) and ≥ 73 months. The study was submitted to and approved by the Human Research Ethics Committee of the Federal University of Viçosa (n. 1,713,903), and all patients signed the Free and Clarified Consent Term (FCCT) and, when applicable, the Term of Assent (TA).

2.2 Sample collection

The samples were collected from November 2016 to September 2017. Data collection was performed by the application of a semi-structured questionnaire based on the International Study of Asthma and Allergies in Childhood (ISAAC), through a duly trained professional. The blood sample was collected by a qualified professional in the Laboratory of Clinical Analysis of the Health Division of UFV, by venous puncture. Two tubes were collected, one with an anticoagulant for hematological analysis and one without anticoagulant, to obtain the blood serum. After clot retraction, the material was centrifuged and aliquot ed for future analyses.

2.3 Evaluation of inflammatory and hematological markers

Serum samples were used for the determination of total IgE and PCRus, using the Turbiquest IgE (Lab Test) and Ultra-Sensitive C Reactive Protein (Bioclin) kits, respectively, following the manufacturers' instructions. The hematological analysis was performed by impedance and the hemogram and leukogram of each patient were determined. Specific reference values were considered for each group.

2.4 Evaluation of oxidative markers

Serum dosage of total antioxidant capacity was based using a modified ferric reduction capacity (FRAP) method (Benzie et al., 1996). Ten μL of sample/standard was added to 220 μL of FRAP solution in polystyrene microplates, followed by
incubation for 30 minutes in the dark. The trolox solution was used as the oxidizing agent, starting from an initial concentration of two mmol.L\(^{-1}\). The readings were performed in a Multiskan GO Microplate Spectrophotometer spectrophotometer (Thermo Scientific) at a wavelength of 570 nm. Concentrations relative to the samples were obtained from the standard curve and are reported in μM.

Thiobarbituric acid reactive substances (TBARS) evaluated lipid peroxidation. The samples were incubated to the thiobarbituric acid solution (1:2), shaken for 10 minutes and subjected to a 90 °C water bath for 40 minutes. Then, 600 μL of n-Butanol was added and the solutions were centrifuged at 1200 x g at room temperature for 5 minutes. The supernatant was used for reading in Multiskan GO Microplate Spectrophotometer (Thermo Scientific) at a wavelength of 532 nm. The absorbances obtained were divided by the molar extinction coefficient of the TBA (\(\varepsilon = 0.156 \, \mu\text{mol.L}^{-1}\) ) and the concentrations of malondialdehyde (MDA) were expressed in nmol.L\(^{-1}\). The measurement of total protein dosage of each of the samples was performed using the bicinchoninic acid (BCA) method. The final concentration of MDA was obtained by the ratio of MDA concentrations to total protein concentrations, and the results are reported as nmol/mg.

The carbonylated protein concentration was based on the 2,4-dinitrophenylhydrazine (DNPH) assay Levine et al., 1990. Absorbance reading was performed at a wavelength of 370 nm in the Multiskan GO Microplate Spectrophotometer (Thermo Scientific). The total protein concentration in the sample was determined by reading the results from the sample in the Genesys 10S UV-Vis spectrophotometer (Thermo Scientific) at 280 nm. The final concentration of the carbonylated protein is reported in nmol/mg of total protein.

2.5 Statistical analysis

The analyzed variables did not present normal distribution in the Shapiro-Wilk test; therefore, we applied the non-parametric Kruskal-Wallis test for multiple
comparisons. The results were expressed as median ± interquartile range. Spearman's distribution and correlation were performed between PCRus and hematological markers, and between total antioxidant capacity (FRAP) and hematological and inflammatory markers. Statistical significance was considered at \( p < 0.05 \). Analyzes were performed using the GraphPad Prism 7.0 program (GraphPad Software, Inc. San Diego, CA).

3 RESULTS

Total immunoglobulin E (IgE) and inflammatory C-reactive protein (PCRus) tests were performed. The patients were divided into three groups according to their age (≤ 36, 37 to 72 and ≥ 73 months). There was no significant difference in IgE and PCR levels among the three groups evaluated (Figure 1A e B).

Figure 1 – Serum IgE (A) and PCRus (B) detection in patients with recurrent wheezing. Volunteers with ≤ 36 months (n = 20); between 37 to 72 months (n = 32); and ≥ 73 months (n = 20). Dotted line (...) represents the reference value for serum IgE detection (100UI/mL) and PCRus (8mg / mL).

Although there was no difference in IgE serum levels, 60% of the lactents had levels above the reference value for this immunoglobulin. The pre-school group...
and the third group had 44% and 30% of the individuals, respectively, with values above the reference. Further, the third group not only presented a lower percentage of individuals with values above the reference but also presented the lowest total IgE serum measurements (Figure 1A). In the detection of PCRus, there was no difference in the percentage of individuals with values above the reference. The lactents, the pre-school and the third groups presented 20%, 22%, and 25%, respectively, of individuals with these values for CRP (Figure 1B).

There was no significant difference in the quantification of total and differential count of leukocytes among the three groups (Table 1). However, the lactents (≤ 36 months) had a neutrophil-lymphocyte ratio lower than the other two groups.

Table 1 – Total and differential count of leukocytes in peripheral blood.

<table>
<thead>
<tr>
<th></th>
<th>≤ 36 (n = 26)</th>
<th></th>
<th>37-72 (n = 34)</th>
<th></th>
<th>≥ 73 (n = 40)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (10^3)</td>
<td>10.1 ± 3.1</td>
<td>13.0 ± 1.6</td>
<td>7.4 ± 2.2</td>
<td></td>
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<tr>
<td>Lymphocytes (10^3)</td>
<td>4.9 ± 2.2</td>
<td>4.6 ± 0.8</td>
<td>2.9 ± 0.8</td>
<td></td>
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<tr>
<td>Neutrophils (10^3)</td>
<td>4.3 ± 1.8</td>
<td>8.6 ± 1.6</td>
<td>3.9 ± 1.6</td>
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<tr>
<td>Eosinophils (10^3)</td>
<td>0.5 ± 0.4</td>
<td>0.2 ± 0.3</td>
<td>0.5 ± 0.3</td>
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<tr>
<td>Monocytes (10^3)</td>
<td>0.3 ± 0.2</td>
<td>3.48 ± 0.1</td>
<td>0.1 ± 0.09</td>
<td></td>
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<tr>
<td>NLR</td>
<td>0.87</td>
<td>1.35</td>
<td>1.35</td>
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SD: standard deviation, NLR: neutrophil-lymphocyte ratio

The correlation between the inflammatory markers was verified by Spearman test. A significant positive correlation between the serum detections of PCRus and leukocytes or neutrophils was observed only in the group of patients with ≥ 73 months of age (Figure 2).
Figure 2 – Spearman correlation (r). Variables PCRus/Leukocytes (A) and PCRus/Neutrophils (B), in patients ≥ 73 months. Statistically significant p < 0.05

The evaluation of total antioxidant capacity in the patients' serum was determined through Ferric Reducing Antioxidant Power (FRAP). The pre-school subjects (37 to 72 months) had a significantly lower serum antioxidant capacity than the other two groups (Figure 3).

Figure 3 – Total antioxidant capacity in the serum of volunteers with recurrent wheezing. Evaluation of iron reduction capacity (FRAP) in volunteers of different ages: < 36 (n = 22), 36 to 72 (n = 42) and ≥ 73 months (n = 39). ** p <0.01 Kurskal-Wallis
Carbonylated protein and lipid peroxidation analysis were performed to verify tissue damage resulting from oxidative stress (Figure 4). The pre-school group (37 to 72 months) presented significantly lower values of carbonylated proteins compared to the other two groups (Figure 4A). Lipid peroxidation showed significantly higher dosages in the group ≥ 73 months compared to the lactents’ group (Figure 4B). For both markers, the older group presented a larger dispersion.

Figure 4 – Quantification markers of tissue damage in volunteers with recurrent wheezing. Evaluation of carbonylated proteins (A) and lipid peroxidation determined by MDA (B) in volunteers of different ages: < 36 (n = 23 in A and n = 22 in B); 36 to 72 (n = 37 in A and n = 42 in B) and ≥ 73 months (n = 37 in A and 36 in B). * p < 0.05 and *** p < 0.005, Kruskal-Wallis

Using the Spearman analysis, the correlation between inflammatory and oxidative markers was determined. Only in the older age group (≥ 73 months), a significant correlation between serum total antioxidant capacity (FRAP) and inflammatory markers (PCRus, Total leukocytes, and Eosinophil) was observed (Figure 5). The FRAP measurements showed a correlation with PCRus r = 0.6081 (Figure 5A), Total leukocytes r = 0.5731 (Figure 5B) and Eosinophil r = 0.5786 (Figure 5C).
DISCUSSION

We report for the first time the inflammatory and oxidative profile of pediatric patients with recurrent wheezing from the Pulmonology Department of the Specialized Care Center in the municipality of Viçosa, Minas Gerais. The children were divided into three groups according to their age: ≤ 36 months (lactents), between 37 to 72 months (pre-school) and ≥ 73 months. We performed an IgE and PCRus analysis for evaluation of inflammatory markers, as well as a leukogram analysis for differential leukocytes count. The serum dosage of FRAP and MDA were used as oxidative stress parameters.

The IgE immunoglobulin is a mediator that plays a central role in the allergic responses of respiratory diseases, such as asthma (Bousquet et al., 2003). In our data, the IgE analysis shows no difference between the groups, although 60% of
the lactents had IgE levels above the values of reference. Lactents at the age of nine months with elevated serum IgE levels have a high risk of developing recurrent wheezing (Martinez et al., 1995). This increased level can be observed in association with both atopic and non-atopic patients (Speight et al., 1983). However, the recurrent wheezing without other allergy symptoms is common and is often associated with viral respiratory infections (Carlsen, 1997). We did not find significant differences between PCRs among the groups. The correlation between serum IgE and PCR in patients is controversial. Although some authors can indicate a positive correlation between them (Bostanci et al., 2016), with high values of PCR in wheezing children from 6 to 36 months of age, a report from another group indicates no correlation between PCR and IgE levels, or even with eosinophil (Livnat et al., 2015).

Systemic inflammation within the first months of life is also a risk factor for episodes of wheezing (Burgess et al., 2016). The hematological profile showed no difference among the groups; however, the infant group had a lower neutrophil-lymphocyte ratio (NLR) than the two older groups. The NLR was reported significantly increased in wheezing and/or asthmatic patients and kept stable in different genders (Gungen & Aydemir et al., 2017, Nacaroglu et al., 2016, Jiang et al., 2017), with a positive correlation with NLR and PCR in other pulmonary diseases (Günay et al., 2014). In our study, it was demonstrated that a positive correlation between PCR to leukocytes and neutrophils occurs only in patients older than 6 years. Patients with recurrent wheezing can present with increased neutrophils counts while lymphocytes decreased markedly, along with an increase in total serum IgE (Jiang et al., 2017). Curiously, we observed that the infant group, which presented a higher percentage of individuals with IgE levels above its reference, also presented the lowest value of the inflammatory NLR.

Oxidative stress plays a major role in airway inflammation that can lead to asthma symptoms such as wheezes. Infections with virus and exposure to tobacco
smoke can also increase ROS generation, hence creating oxidative stress (Singh et al., 2016). We found significant differences in serum detections of FRAP and carbonylated proteins only in the pre-school group. This group had significantly lower detection of carbonylated proteins compared to the other two groups that indicate lower oxidative damage in proteins. However, the value of FRAP was also significantly lower in this group, which indicates that the wheezes, and a probable association with asthma, affects the disease's pathogenesis through a reduction in the antioxidant capacity. MDA was found to be significantly increased in children older than six years compared to younger groups. There is a positive correlation between MDA, the age of asthmatic patients, and the duration of the disease, whereas there is an age-related increase oxidative damage (Radović et al., 2013, Singh et al., 2016).

There was a positive correlation between serum FRAP and inflammatory markers (PCR, leukocyte, and eosinophil) only in the children above 73 months of age. The antioxidant status in the airway is established by immune cells through the release of antioxidant enzymes (i.e. myeloperoxidase and superoxide dismutase) (Rahman et al., 2006), which can be associated to the correlation of FRAP with immune cells observed in our data. In this way, recurrent wheezing patients, older than 6 years, have a relationship between inflammation and other immune responses. Despite this, the PCR level had no significant difference between the groups. High levels of CRP and FRAP are observed in asthmatic patients and is linked with increased cellular activation (Akiibinu et al., 2014, Sahoo et al., 2009).

Oxidative stress and inflammatory markers seem to play a role in wheezing children. Our data showed different alterations in these measures among the subjects. It was observed that there was a reduction in FRAP and carbonylated protein only in preschool children. A positive correlation between oxidative markers and immune cells show a significant difference only in children above six
years old. These may contribute to the different phenotypes of wheezing episodes in children, though the profiles presented vary.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

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