

**Warifteine therapeutic treatment reduced leukocyte recruitment and anxiety-like response in ovalbumin-induced allergic pulmonary inflammation****Tratamento terapêutico com warifteína reduz recrutamento de leucócitos e resposta semelhante á ansiedade na inflamação pulmonar alérgica induzida por ovalbumina**

DOI:10.34117/bjdv6n9-124

Recebimento dos originais: 08/08/2020

Aceitação para publicação: 08/09/2020

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**ABSTRACT**

Pulmonary inflammation plays a fundamental role in the pathophysiology of allergic asthma, which is characterized by lower airway obstruction, bronchial hyperresponsiveness, tissue remodeling, recruitment of inflammatory cells, with a predominance of eosinophils, in addition to behavioral disorders such as anxiety. The aim of this study was to evaluate the therapeutic effect of the alkaloid warifteine, from the medicinal plant *Cissampelos sympodialis*, on anxiety-like behavior, respiratory frequency and leukocyte recruitment in an experimental model of allergic pulmonary inflammation. Swiss female mice were sensitized and challenged with ovalbumin (OVA) throughout the experimental protocol. The animals were treated orally with warifteine (2 mg / kg), subcutaneously with dexamethasone (2 mg / kg) or intraperitoneally with diazepam (1 mg / kg), 1 h after the OVA-challenges. On the last day of the antigenic challenge, the mice were tested for behavior using the Elevated Plus Maze (EPM) and for respiratory rate using full body plethysmography. The following day, the mice were euthanized to collect the bronchoalveolar lavage fluid (BALF) and leukocyte count. The data obtained showed that OVA-sensitization induced a behavior similar to anxiety in mice since the EPM test showed that the OVA group increased the number of entries and the time spent in the closed arms (CA) of the apparatus and reduced these parameters in the open arms (OA) compared to the Salina group. Warifteine treatment reversed both parameters analyzed, increasing the time spent ( $p < 0.0001$ ) and number of entries ( $p < 0.01$ ) in OA, decreasing the time spent ( $p < 0.01$ ) and number of entries ( $p < 0.0001$ ) in the CA, similarly to dexamethasone and diazepam standard drugs. Warifteine also reduced the respiratory rate ( $p < 0.01$ ) compared to the OVA group. The behavioral and breathing changes of the tested animals showed a relationship with the increase in the total and differential inflammatory leukocyte number in the OVA group compared to the Saline group. Therapeutic treatment with warifteine decreased the inflammatory process, reducing the number of total cells ( $p < 0.0001$ ) dependent of eosinophils and neutrophils numbers ( $p < 0.001$ ), as well as the percentage of eosinophils ( $p < 0.0001$ ). These data show that therapeutic treatment

with warifteine is able to inhibit anxiety-like behavior and respiratory rate, due to a mechanism related to the inhibition of eosinophilic migration in an experimental model of allergic pulmonary inflammation.

**Keywords:** Alkaloid, Airway disease, Psychological disorder.

## RESUMO

A inflamação pulmonar desempenha papel fundamental na fisiopatologia da asma alérgica, que se caracteriza por obstrução das vias aéreas inferiores, hiperresponsividade brônquica, remodelamento tecidual, recrutamento de células inflamatórias, com predomínio de eosinófilos, além de distúrbios comportamentais como a ansiedade. O objetivo deste estudo foi avaliar o efeito terapêutico do alcalóide warifteína, da planta medicinal *Cissampelos sympodialis*, sobre o comportamento semelhante à ansiedade, a frequência respiratória e o recrutamento de leucócitos em um modelo experimental de inflamação pulmonar alérgica. Camundongos fêmeas Swiss foram sensibilizados e desafiados com ovalbumina (OVA) ao longo do protocolo experimental. Os animais foram tratados por via oral com warifteína (2 mg / kg), por via subcutânea com dexametasona (2 mg / kg) ou intraperitoneal com diazepam (1 mg / kg), 1 h após os desafios com OVA. No último dia do desafio antigênico, os camundongos foram testados quanto ao comportamento usando o Labirinto em Cruz Elevado (*do inglês*, EPM) e quanto à frequência respiratória usando pletismografia de corpo inteiro. No dia seguinte, os camundongos foram sacrificados para coleta do fluido do lavado broncoalveolar (*do inglês*, BALF) e contagem de leucócitos. Os dados obtidos mostraram que a sensibilização com OVA induziu um comportamento semelhante à ansiedade em camundongos, uma vez que o teste de EPM mostrou que o grupo OVA aumentou o número de entradas e o tempo de permanência nos braços fechados (*do inglês*, CA) do aparelho e reduziu esses parâmetros no braços abertos (*do inglês*, OA) em comparação com o grupo Salina. O tratamento com warifteína reverteu ambos os parâmetros analisados, aumentando o tempo de permanência ( $p < 0,0001$ ) e o número de entradas ( $p < 0,01$ ) no OA, diminuindo o tempo de permanência ( $p < 0,01$ ) e o número de entradas ( $p < 0,0001$ ) no CA, semelhantemente aos medicamentos padrão dexametasona e diazepam. Warifteína também reduziu a frequência respiratória ( $p < 0,01$ ) em comparação com o grupo OVA. As alterações comportamentais e respiratórias dos animais testados mostraram uma relação com o aumento no número total e diferencial de leucócitos inflamatórios no grupo OVA em relação ao grupo Salina. O tratamento terapêutico com warifteína diminuiu o processo inflamatório, reduzindo o número de células totais ( $p < 0,0001$ ) dependentes de eosinófilos e neutrófilos ( $p < 0,001$ ), bem como a porcentagem de eosinófilos ( $p < 0,0001$ ). Esses dados mostram que o tratamento terapêutico com warifteína é capaz de inibir o comportamento semelhante à ansiedade e a frequência respiratória, devido a um mecanismo relacionado à inibição da migração eosinofílica em um modelo experimental de inflamação pulmonar alérgica.

**Palavras-chave:** Alcaloide, Doenças das vias aéreas. Desordem psicológica.

## 1 INTRODUCTION

Allergic asthma represents a worldwide public health problem affecting more than 350 million people worldwide [1,2]. This is a chronic inflammatory disease of the lower airways, characterized by overproduction of mucus, airway hyperresponsiveness, tissue remodeling and recruitment of inflammatory cells, with a predominance of eosinophilic inflammation [3].

Symptoms vary over time in terms of occurrence, frequency and intensity and generally include chest tightness, wheezing, coughing, shortness of breath and reduced lung function [4].

The development of anxiety symptoms has been described in asthmatic patients and in experimental models of asthma [5-7], which indicates a close relationship between the components of the Immune System and the Central Nervous System. The study of neuroimmunomodulation shows a bidirectional interaction between the immune system and areas of the nervous system involved in emotional processing (commonly referred to as "limbic system"), demonstrating that changes in immune responses, such as those that occur in allergic lung inflammation, are capable of modulating neuronal activity and, consequently, emotions and behavior [8,9]. In addition, neuropeptides and neurotransmitters derived from neurons regulate the functions of immune cells, while the inflammatory mediators produced by immune cells increase neuronal activation [10]. In some studies, it has been shown that neuropeptides such as corticotropin-releasing hormone, substance P and neurotensin promote neuroimmune stimulation of mast cells, contributing to allergic and inflammatory processes in patients with asthma [11]. These data provide the basis for understanding the mechanisms that lead to psychiatric asthma symptoms.

Asthma and anxiety disorder relationship has significant influences on the treatment of patients, worsening symptoms, precipitating attacks, causing a reduction in asthma control and, thus, reducing patient quality of life [12]. In this way, psychosocial conditions constitute a barrier to asthma control and raise the importance of managing anxiety for the treatment of asthmatic patients [6,7].

In addition, in humans it is recognized there is a link between respiratory changes and anxiety disorders, and respiratory symptoms constitute diagnostic parameters for various anxiety-related conditions [13]. Experimental studies demonstrate the association between breathing and anxiety in rodents, in which respiratory parameters, especially respiratory rate, are influenced by aversive stimuli and novelty [14,15]. Thus, given the importance of breathing in human anxiety, pre-clinical research aims to investigate the respiratory function in animal models of anxiety.

Pharmacological treatment for allergic asthma and anxiety is made by the combination of corticosteroids,  $\beta$ 2-adrenergic agonists and benzodiazepines. However, the use of these drugs is often associated with the appearance of undesirable side effects such as tachycardia and tremors due to the use of  $\beta$ 2-adrenergic agonists [16,17], in addition to the drowsiness and chemical dependence attributed to the use of benzodiazepines [18]. Thus, there is a growing need for the development of new drugs that combine antiasthmatic and anxiolytic properties.

Medicinal plants and their bioactive molecules are alternatives to conventional therapies for many diseases. *Cissampelos sympodialis* Eichler (Menispermaceae) is an endemic species of Northeastern Brazil, whose leaves and roots are used in indigenous and popular medicine in the treatment of several inflammatory diseases, including asthma [19,20]. The bisbenzylisoquinoline alkaloid warifteine, isolated from *C. sympodialis* represents the major compound of the plant and its properties have been reported such as anti-inflammatory, anti-allergic, immunomodulatory and psychoactive in several experimental models [21-31]. Thus, the main objective of this study was to evaluate the oral therapeutic treatment with the alkaloid warifteine in anxiety-like response, respiratory rate and leukocyte recruitment in an experimental model of allergic pulmonary inflammation.

## **2 MATERIAL AND METHODS**

### **2.1 ANIMALS**

Female Swiss mice aged between 6 and 8 weeks and body weight between 20 and 30 g were used. The animals were kept in polypropylene cages at a temperature of  $25 \pm 2^\circ\text{C}$  and subjected to a 12-hour light and dark cycle with free access to water and a controlled diet based on pellet food (PURINA) throughout the trial period. The mice were supplied by the Central Animal House Professor Thomas George of the Research Institute of Drugs and Medicines of the Federal University of Paraiba (IPeFarM / UFPB / João Pessoa, PB). Experimental protocols were carried out in accordance with the recommendations of the Committee for Experimentation in Animal Research at UFPB (CEUA / UFPB). Animals were euthanized by an overdose of anesthetics (xylazine 48 mg / kg + ketamine 360 mg / kg) administered intramuscularly (i.m.). All experimental procedures were analyzed and approved by CEUA / UFPB, under certificate n°4773180418 (ID 000296).

### **2.2 THE ALKALOID WARIFTEINE**

The alkaloid warifteine was isolated from the hydroalcoholic extract of the roots and leaves of *Cissampelos sympodialis* and kindly provided by Prof. Dr. José Maria Barbosa Filho from the Federal University of Paraiba, João Pessoa, PB, Brazil.

### **2.3 OVALBUMIN-INDUCED ALLERGIC LUNG INFLAMMATION**

The experimental model of allergic pulmonary inflammation induced by ovalbumin (OVA) consisted of sensitizing female Swiss mice on days 0 and 10 with intraperitoneal injection (ip) of

0.2 mL of a suspension containing 10 µg of grade V OVA (SIGMA Chemical, St. Louis, MO) and 0.2 g of Al (OH) 3 (VETEC, Rio de Janeiro, RJ) in saline [32]. In the period between days 19 to 24 after sensitization, the animals were submitted to allergenic challenge with 5% OVA aerosol grade II (SIGMA Chemical, St. Louis, MO) in saline solution. Each challenge was performed for 20 minutes daily in a closed chamber, under a continuous aerosol flow, with the aid of an ultrasonic nebulizer. In the following day, the animals were euthanized by anesthetic overdose and the trachea was surgically exposed and cannulated for later collection of the Bronchoalveolar Lavage (BALF). Samples were processed to microscopy slides preparation and total and differential leukocyte count.

#### 2.4 TREATMENT

The animals were distributed into five experimental groups (n = 5). The Saline, OVA and OVA + warifteine (2 mg / kg, vo) or OVA + dexamethasone (2 mg / kg, sc) (DECADRON, Achê®, 0.2 mg / mL) or OVA + diazepam (1 mg / kg, ip) (Diazepam, Teuto®, 5mg / mL). Oral treatments were given 1 hour after (therapeutic treatment) each OVA aerosol challenge for 6 consecutive days according to the experimental protocol.

#### 2.5 BEHAVIORAL ANALYSIS

Immediately after the last allergenic challenge and treatment, animals were evaluated for the presence of anxiety-like behavioral signs, using the Elevated Plus Maze (EPM) test. The device used in the EPM test was made of gray acrylic material (Insight®, São Paulo, Brazil) and had two open arms (30 cm x 5 cm x 15 cm) and two closed arms (30 cm x 5 cm x 15 cm) ) that radiated from a central platform (5 x 5 cm), presenting the shape of a cross raised 38.5 cm from the floor and placed inside an attenuated sound room. EPM test was started by placing the animal on the central platform of the labyrinth, facing an open arm according to the experimental protocol. Then, the number of entries and the time spent in the open and closed arms of the device was recorded for a period of 5 minutes for each animal individually.

#### 2.6 RESPIRATORY RATE ANALYSIS

After conducting the behavioral analysis, the animals were submitted to a measurement of respiratory frequency using the plethysmography technique in a full body chamber for small animals, adapted from the literature [33]. The procedure consists of inserting the animal in a closed system at the entrance of gases. Pressure fluctuations within the system were considered due to the temperature difference between the inspired gas (~ 25 C°) and the expired gas (~ 37 C°) for the



analysis of ventilatory parameters. The procedure consisted of opening the chamber for placing the animal, closing it, interrupting the air flow and performing a respiratory rate measurement for a period of about 3 minutes. The oscillations caused by the animal's breathing were captured by a device connected to the chamber that contains a differential pressure transducer (ML141 Spirometer, PowerLab, ADInstruments). The signal was then sent to the data analysis acquisition system (LabChart TM Pro, PowerLab, ADInstruments) that amplifies the signals and quantifies the amplitude and frequency of the respiratory signals. The volume calibration was obtained during each experiment, by injecting a known volume of air (0.2 mL) into the animal's chamber using a graduated syringe. The system allowed the recording of respiratory rate (RF) measurements in Volts (V) which was later analyzed in cycles per minute (cpm). The RF quantification was performed by removing from the total record of approximately 3 minutes a stretch corresponding to 10 seconds free of noise and representative of the pattern of the generated response.

## 2.7 BRONCHOALVEOLAR LAVAGE FLUID (BALF)

On the 25th day of the experimental protocol, the animals were anesthetized, the tracheas were exposed with the aid of forceps and surgical scissors, the lobes of the thyroid gland were removed and then an IV-18G polyurethane peripheral catheter (Descarpac®) was inserted into the trachea and a syringe containing 1 mL of HBSS buffer was connected to the catheter for lung washing and BALF collection. OAL samples were transferred to eppendorf tubes and stored on ice, to preserve cell viability and later count the total and differential cell number.

## 2.8 BALF CELL COUNT

BALF was diluted 1:4 in Turk's solution (VETEC, Rio de Janeiro, RJ) and then it was taken to the hemocytometric chamber (NeuOAUer) for total cells counting under an optical microscope (40 X - BX40, OLYMPUS). Then, the tubes were centrifuged (CR422, JONAM centrifuge) at 1000 rpm, 4°C, 5 minutes, pellets were resuspended in 500µL of HBSS - / - and centrifuged in cytopspin type cytocentrifuge (FANEM, São Paulo, SP, Brazil Mod 2400). The slides obtained were fixed and stained by the panotic method (Kit Panótico, Renylab). The differential cell count was performed by optical microscopy. Each slide was traversed until the counting of 100 cells, using the immersion objective (100x). From the counting of the differential cells, the percentage of eosinophils was determined.

**2.9 STATISTICAL ANALYSIS**

All data were analyzed with the aid of the Graph Pad Prism © program version 7.0 (GraphPad Software, San Diego, CA, U.S.A.). The results obtained were expressed as mean  $\pm$  standard error of the mean (e.p.m.) and analyzed statistically using the one-way ANOVA followed by the Tukey test, where  $p$  values  $<0.05$  were considered significant. Values of +  $p <0.05$ ; ++  $p <0.01$  and +++  $p <0.0001$  were considered significant when compared with the basal group, and \*  $p <0.05$ ; \*\*  $p <0.01$  and \*\*\*  $p <0.0001$  were considered significant when compared with the OVA group.

**3 RESULTS****3.1 EFFECT OF WARIFTEINE ON ANXIETY-LIKE BEHAVIOR IN OVALBUMIN-SENSITIZED MICE**

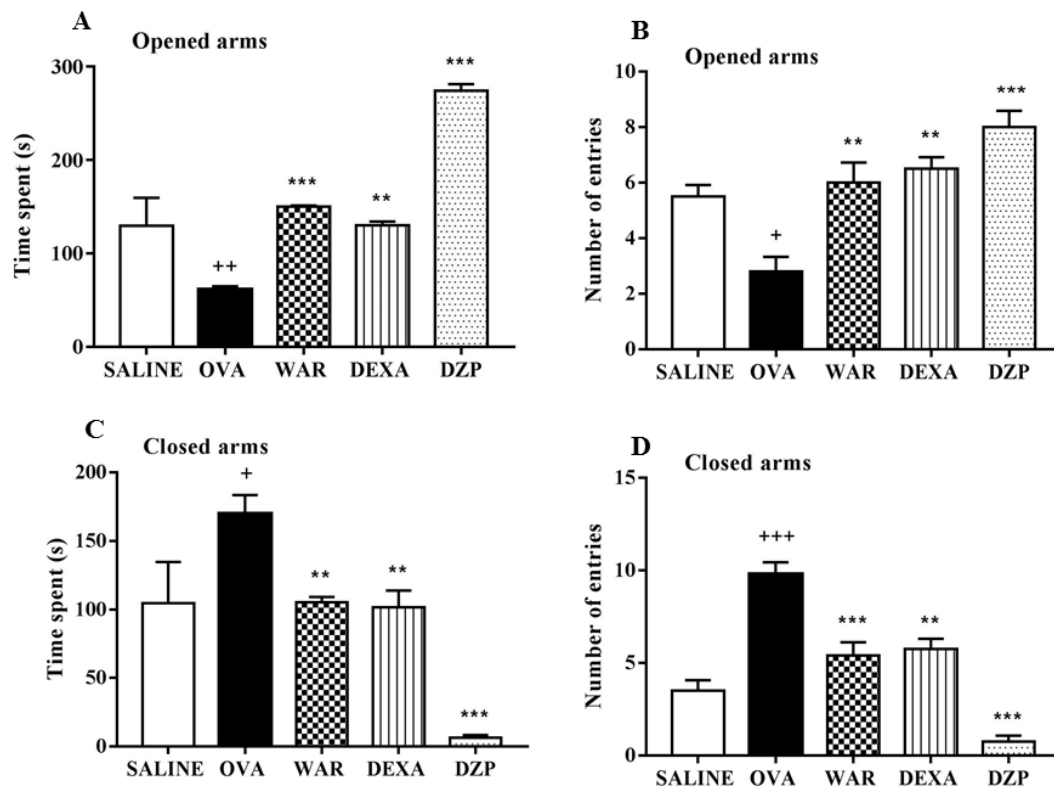
Mice from the OVA group showed a significant reduction in the time spent, in seconds, in the open arms (OA) ( $61 \pm 3.84 - p <0.01$ ) as well as in the number of entries in the OA of the EPM ( $2.8 \pm 0.53 - p <0.05$ ) when compared to the Saline group ( $129.7 \pm 29.9$  and  $5.5 \pm 0.42$ , respectively) (Figure 1A, B). Treatment with warifteine significantly increased both the time spent in OA ( $149.8 \pm 1.45 - p <0.0001$ ) and the number of entries in OA ( $6 \pm 0.73 - p <0.01$ ) compared to the OVA group. Besides, treatments with the standard anti-inflammatory drug dexamethasone, or with the standard anxiolytic drug diazepam induced significant increases in the time spent in the OA ( $130 \pm 4.11 - p <0.01$  and  $274 \pm 7.28 - p <0.0001$ , respectively), and in the number of entries in the OA ( $6.5 \pm 0.42 - p <0.01$  and  $8 \pm 0.59 - p <0.0001$ , respectively) as compared to animals of the OVA group.

On the other hand, the animals in the OVA group showed a significant increase in the time spent in the closed arms (CA) ( $170 \pm 13.5 - p <0.05$ ) as well as in the number of entries in the CA of the EPM ( $9.8 \pm 0.64 - p <0.0001$ ) when compared to the Saline group ( $104.5 \pm 30.3$  and  $3.5 \pm 0.56$ , respectively) (Figure 1C, D).

Warifteine treatment was able to significantly reduce the time spent in the CA ( $105.4 \pm 3.87 - p <0.01$ ), as well as the number of entries in the CA ( $5.4 \pm 0.71 - p <0.0001$ ), compared to the OVA group. In addition, treatments with the standard anti-inflammatory drug dexamethasone or with the standard anxiolytic drug diazepam promoted a significant reduction both in the time spent in the CA ( $101.5 \pm 12.4 - p <0.01$  and  $6.3 \pm 2, 01 - p <0.0001$ , respectively), and the number of entries in the CA ( $5.7 \pm 0.55 - p <0.01$  and  $0.75 \pm 0.31 - p <0.0001$ , respectively) when compared to animals in the OVA group.



Figure 1. Effect of warifteine on anxiety-like behavior in ovalbumin-sensitized mice

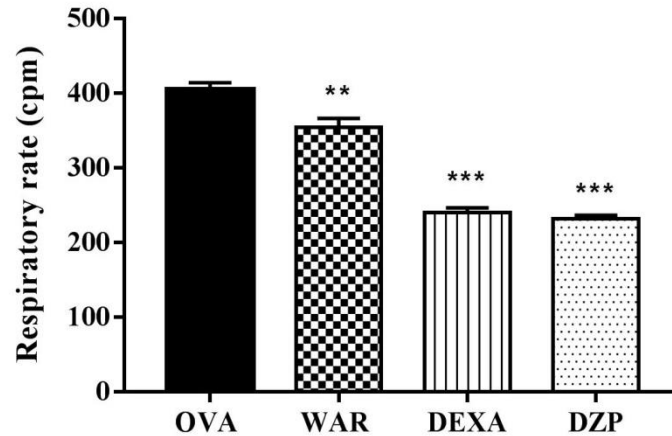


Female Swiss mice ( $n = 5$ ) were sensitized and challenged with Ovalbumin (OVA) and treated with the alkaloid warifteine (WAR - 2 mg / kg), with the standard anti-inflammatory drug Dexamethasone (DEXA - 2 mg / kg) or with the drug- anxiolytic pattern Diazepam (DZP - 1 mg / kg) 1 hour after the challenges. Immediately after the last challenge and treatment, the animals were taken to the EPM test. A. Time spent in open arms (OA). B. Number of entries in the open arms (OA). C. Time spent in closed arms (CA). D. Number of entries in the closed arms (CA). The results were expressed as mean  $\pm$  s.p.m. (One-way ANOVA followed by the Tukey test). +  $p < 0.05$ ; ++  $p < 0.01$ ; +++  $p < 0.0001$ , compared to the Saline group; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.0001$  compared to the OVA group.

### 3.2 EFFECT OF WARIFTEINE ON THE RESPIRATORY RATE OF OVALBUMIN-SENSITIZED MICE

Mice treated with warifteine showed a reduction in respiratory rate ( $354.3 \pm 12.2 - p < 0.01$ ) compared to mice in the OVA group ( $405.3 \pm 9, 09$ ). Also, treatment with dexamethasone or diazepam reduced the respiratory rate of the animals ( $239.8 \pm 6.57$  and  $231.3 \pm 5.04 - p < 0.0001$  respectively) compared to the OVA group (Figure 2).

Figure 2. Effect of warifteine on the respiratory rate of ovalbumin-sensitized mice



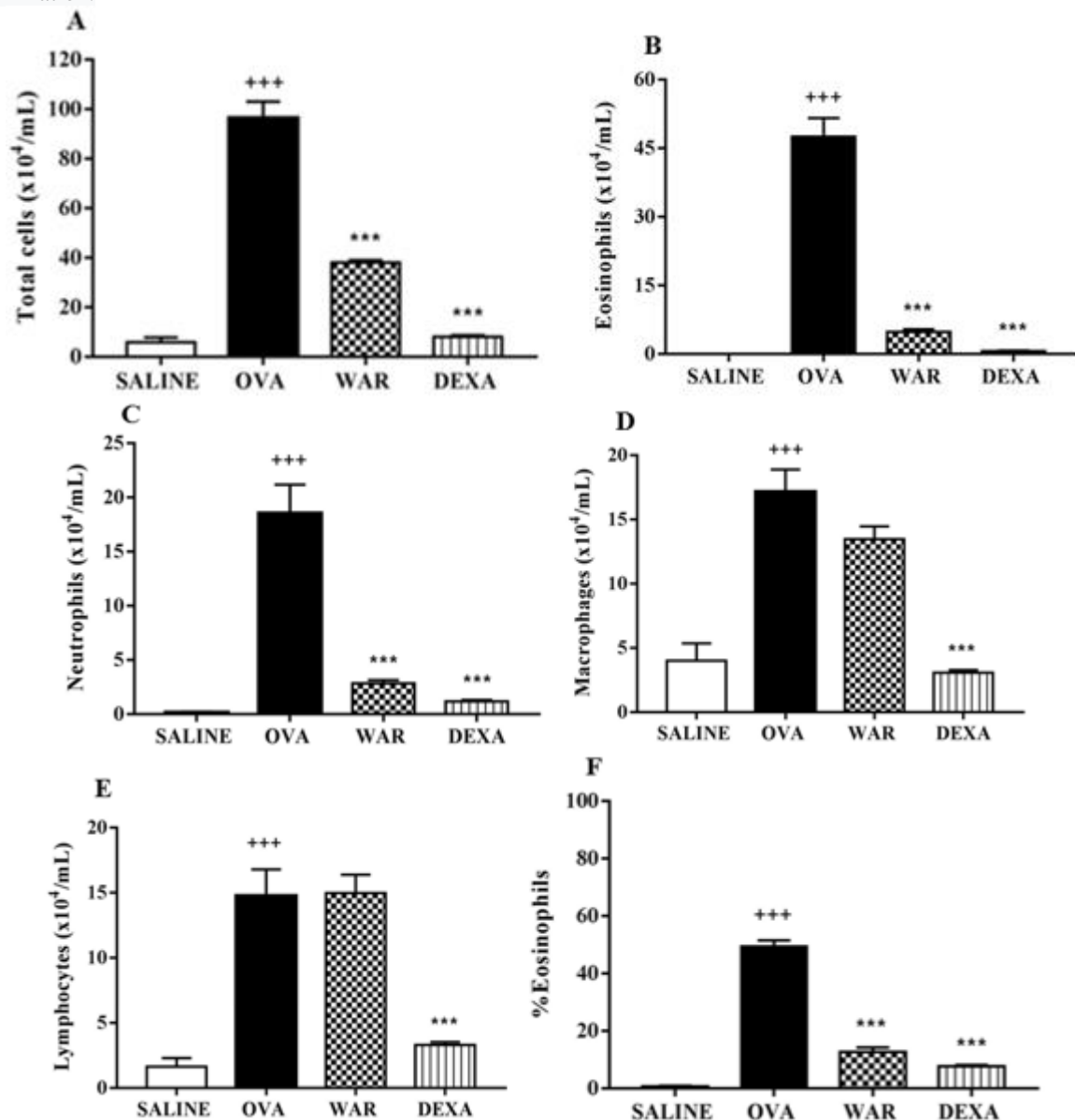
Female Swiss mice (n = 5) were sensitized and challenged with Ovalbumin (OVA) and treated with the alkaloid warifteine (WAR - 2 mg / kg), with the standard anti-inflammatory drug Dexamethasone (DEXA - 2 mg / kg) or with the drug- anxiolytic pattern Diazepam (DZP - 1 mg / kg) 1 hour after the challenges. Immediately after the EPM test, the animals were taken to a full body plethysmograph for analysis of respiratory rate. The results were expressed as mean  $\pm$  e.p.m (one-way ANOVA followed by the Tukey test). \*\* p <0.01; \*\*\* p <0.0001 compared to the OVA group.

### 3.3 EFFECT OF WARIFTEINE TREATMENT ON LEUKOCYTE RECRUITMENT TO BALF

Animals in the OVA group showed a significant increase in the leukocyte recruitments ( $10^4$  / mL) into the bronchoalveolar cavity compared to the Saline group (Fig. 3A,  $96.33 \pm 6.85$  - p <0.0001 vs  $5, 83 \pm 2.04$ ). Differential cell count revealed that this increase was due to the influx of eosinophils (Fig. 3B,  $47.51 \pm 4.12$  - p <0.0001 vs  $0.018 \pm 0.006$ ), neutrophils (Fig. 3C,  $18.56 \pm 2.64$  - p <0.0001 vs  $0.15 \pm 0.06$ ), macrophages (Fig. 3D,  $17.18 \pm 1.73$  - p <0, 0001 vs  $4.01 \pm 1.34$ ) and lymphocytes (Fig. 3E,  $14.75 \pm 2.04$  - p <0.0001 vs  $1.64 \pm 0.64$ ). The OVA group showed a significant increase in the percentage (%) of eosinophils in the bronchoalveolar cavity compared to the Saline group (Fig. 3F,  $49.33 \pm 2.17\%$  - p <0.0001 vs  $0.66 \pm 0.21\%$ ).

Therapeutic treatment with warifteine, as well as dexamethasone, was able to significantly reduce the number of total cells (Fig. 3A,  $38 \pm 1$ ;  $8.16 \pm 0.60$  - p < 0, 0001, respectively) when compared to the OVA group. Warifteine treatment was able to significantly reduce the number of eosinophils and neutrophils (Fig. 3B-C,  $4.80 \pm 0.56$  and  $2.84 \pm 0.29$  - p <0.001, respectively) in the BALF when compared to the OVA group. Treatment with dexamethasone decreased the number of eosinophils (Fig. 3B,  $0.63 \pm 0.076$  - p <0.0001), neutrophils (Fig. 3C,  $1.17 \pm 0.12$  - p <0.0001), lymphocytes (Fig. 3D,  $3.05 \pm 0.21$  - p <0.0001) and macrophages (Fig. 3E,  $3.30 \pm 0.21$  - p <0.0001) in the BALF when compared to the OVA group as expected. No changes were observed regarding the effect of warifteine on macrophage and lymphocyte count. Warifteine and dexamethasone also was able to reduce the percentage of eosinophils in the BALF ( $12.67 \pm 1.54\%$ ;  $7.66 \pm 0.49\%$  - p <0, 0001, respectively) when compared to the OVA group.

Figure 3. Effect of warifteine on the leukocyte recruitment into the BALF on experimental allergic pulmonary inflammation.



Female mice (n = 5) were sensitized and challenged with Ovalbumin (OVA) and treated with the alkaloid warifteine (WAR - 2 mg / kg) or the standard drug Dexamethasone (DEXA - 2 mg / kg) 1 hour after the challenge. The Bronchoalcoholic Lavado (BALF) was collected 24h after the last challenge and the migration of total and differential cells was quantified in standard optical microscopy. A) Total Cells; B) Eosinophils; C) Neutrophils; D) Macrophages; E) Lymphocytes; F) Percentage of Eosinophils. The results were expressed as mean  $\pm$  e.p.m (one-way ANOVA followed by the Tukey test). +++ p < 0.0001, compared to the Saline group; \*\*\* p < 0.0001 compared to the OVA group.

#### 4 DISCUSSION

It has been demonstrated the effect of the therapeutic treatment with the alkaloid warifteine on anxiety-like behavior, respiratory rate and leukocyte recruitment in an experimental model of allergic pulmonary inflammation.

Sensitization protocol induced an increase in the time spent and number of entries of the mice in the closed arms of the EPM and the opposite response in the open arms of the apparatus

indicating an OVA-anxiogenic effect. Conversely, treatment with the standard anxiolytic drug diazepam reversed the anxiety-like response by promoting a significant inhibition in the parameters mentioned above. These findings corroborate with a previous study carried out by our research group which showed similar response in OVA-sensitized mice [34]. Therapeutic treatment with the alkaloid warifteine was able to reverse the anxiety-like behavior induced by OVA similar to diazepam indicating that warifteine assumes behavioral characteristics of an anxiolytic drug. Recent study has shown the effectiveness of prophylactic treatment with warifteine on the behavioral response [35].

Chronic allergic diseases have a considerable quality of life impact. Studies show that asthmatic patients have a higher incidence of behavioral disorders, such as depression and anxiety, regardless of the level of asthma severity [36]. These behavioral conditions directly interfere in the management of asthma and have been associated with low adherence to treatment and increased morbidity and mortality [37]. In search of a better understanding of the mechanisms involved in the anxiety-like response, EPM test has been widely used as one of the main murine models in the search for new anxiolytic drugs as the well-established benzodiazepines [38].

The allergic process has been reported as an important factor in altering the respiratory rate of asthmatics and experimental animals [39,35]. Pre-clinic studies demonstrated the association between breathing and anxiety in animals, in which respiratory parameters, especially the respiratory rate, are influenced by aversive stimuli and novelty [14,15]. In the present study, we observed the therapeutic treatment with warifteine diminished the respiratory rate when compared to OVA-sensitized mice similarly to dexamethasone and diazepam drugs. These data allow us to understand that both allergic diseases and anxiety lead to respiratory disorders.

Pulmonary inflammation model also caused a significant leukocyte recruitment into the bronchoalveolar cavity of the animals. Importantly, the robust leukocyte recruitment into the sensitized-mice airways was an average of 50% eosinophils and it has been previously described that eosinophil percentage in the BALF reaches values between 40-60% of inflammatory cells in OVA-sensitized mice [40]. In fact, several studies have demonstrated the relevance of eosinophilic inflammation and its crucial role in the asthma pathophysiology [41-43]. Then, therapeutic treatment with warifteine strongly reduced the migration of eosinophils into the BALF similarly to the anti-inflammatory drug dexamethasone. These current data are in accordance with previous studies with bisbenzylisoquinoline alkaloids as warifteine and curine [23, 44].

Accordingly, enhanced count for neutrophils, lymphocytes and macrophages into the inflamed airways were observed in this work and the presence of these cell populations has been

described into the asthmatic lung tissue especially during symptomatic manifestations, where they have a harmful pro-inflammatory potential [45]. In this study, therapeutic treatment with warifteine also inhibited neutrophil migration but not lymphocytes and macrophages into the BALF. This phenomena might be explained considering the warifteine administration route and its different effect on the leukocyte recruitment as observed in a previous study which revealed macrophages and lymphocytes reduced numbers into the BALF in OVA-sensitized mice treated by nasal instillation with warifteine [46].

In conclusion, the therapeutic treatment with the alkaloid warifteine, isolated from the medicinal plant *Cissampelos sympodialis* showed an inhibitory effect on anxiety-like behavior, respiratory rate and eosinophilic recruitment in an experimental model of allergic pulmonary inflammation. These findings represent a potential for the development of a monotherapy to act both at the central and peripheral levels to the treatment of asthma and psychological disorders.

#### **ACKNOWLEDGMENTS**

This study was financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (ID: 304772/2014-3; 429767/2016-1, VAB), Paraíba State Research Foundation (FAPESQ, ID: 007/2019 FAPESQ-PB-MCT/CNPq) and The Institutional Program of Scientific Initiation Scholarships from Federal University of Paraíba – UFPB.

#### **CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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