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Sakuranetin reverses vascular peribronchial and lung parenchyma remodeling in a murine model of chronic allergic pulmonary inflammation

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ABSTRACT

Background and purpose: Asthma is a disease of high prevalence and morbidity that generates high costs in hospitalization and treatment. Although the airway is involved in the physiopathology of asthma, there is also evidence of the importance of vascular and lung parenchyma inflammation and remodeling, which can contribute to the functional pulmonary alterations observed in asthmatic patients. Our aim was to evaluate treatment using sakuranetin, a flavone isolated from the twigs of *Baccharis retusa* (Asteraceae), on vascular and lung parenchyma alterations in an experimental murine model of asthma.

Methods: Male BALB/c mice were subjected to a sensitization protocol with ovalbumin for 30 days and were treated with or without sakuranetin (20 mg/kg/mice) or dexamethasone (5 mg/kg/mice); then, the lungs were collected for histopathological analysis. We evaluated extracellular matrix remodeling (collagen and elastic fibers), inflammation (eosinophils and NF-κB) and oxidative stress (8-isoprostane) in the pulmonary vessels and lung parenchyma. The thickness of the vascular wall was quantified, as well as the vascular endothelial growth factor (VEGF) levels.

Results: We demonstrated that sakuranetin reduced the number of eosinophils and elastic fibers in both the pulmonary vessels and the lung parenchyma, probably due to a reduction of oxidative stress and of the transcription factor NF-κB and VEGF levels in the lung. In addition, it reduced the thickness of the pulmonary vascular wall. The treatment had no effect on the collagen fibers. In most of the parameters, the effect of sakuranetin was similar to the dexamethasone effect.

Conclusions and implications: Sakuranetin had anti-inflammatory and antioxidant effects, preventing vascular and distal parenchyma changes in this experimental model of asthma.

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1. Introduction

Inflammation is the most important pathogenic factor for asthma and is the result of complex interactions among inflam-

matory cells, mediators and structural airway cells. Inflammation is present even in asymptomatic patients. The inflammatory response has special features, including eosinophil infiltration, mast cell degranulation, interstitial injury of the airway wall, activation of Th2 lymphocytes that produce cytokines such as IL-4, IL-5 and IL-13, increased vascular permeability and vasodilation, among others, which are responsible for initiating and maintaining the inflammatory process (GINA, 2014).

Chronic lung inflammation may generate a lung repair process, known as remodeling, that is mainly characterized by peribronchial

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fibrosis, smooth muscle hypertrophy, epithelial changes, and the proliferation of fibroblasts with the subsequent deposition of collagen and elastic fibers and is also associated with a decrease in lung function, which is observed in asthma patients and in experimental models (Prado et al., 2011; Mauad et al., 2000).

Most studies of asthma end up focusing on only airway changes. However, it is agreed that it may affect tissue remodeling, including vascular changes. In addition, it is known that there is an extensive involvement of the lung parenchyma in asthma because the peripheral lung tissue responds to bronchoconstrictor stimuli (Dolhnikoff et al., 1998; Righetti et al., 2014).

The gold-standard treatment recommended for asthmatic patients is the use of corticosteroids. However, this is not always effective in preventing or in reversing lung remodeling, particularly in the lung parenchyma and peribronchial vessels (Bergeron et al., 2005). There are still a small percentage of patients with asthma who have difficulty controlling and not responding to treatment with systemic and/or inhaled corticosteroid.

Recent studies suggest that low toxic flavonoids may exert potent anti-inflammatory, antiallergic, antioxidant and antiproliferative effects on allergic inflammatory diseases (Kimata et al., 2000; Shichijo et al., 2003; Teixeira et al., 1995; Hirano et al., 2004; Dornas et al., 2007; Havsteen, 2002). However, the underlying biological molecular mechanisms of these effects have not been fully elucidated, and several lines of evidence suggest the existence of indeterminate molecular targets (Havsteen, 2002; Lotito and Frei, 2006; Middleton et al., 2000).

Among the various plant species used in folk medicine are those belonging to the family Asteraceae, including *Baccharis*, which are shrubs with a wide dispersion, mainly in Brazil, Argentina, Colombia, Chile and Mexico. This plant species has been studied because of its chemical composition and biological activity, which are recognized as being important for medicine in the treatment and prevention of various diseases (Verdi et al., 2005). Over 500 species of *Baccharis* are described, among which, *B. retusa*, which is from twigs, is mainly composed of the flavonoid sakuranetin (Grecco et al., 2012).

Because the recommended treatment with corticosteroids for asthma patients has no proven effect on vascular remodeling and distal lung parenchyma, it is important to develop further studies aimed at seeking new therapeutic targets. Given its anti-inflammatory property and that fact that it is shown to have beneficial effects in the airways of a murine model of asthma (Toledo et al., 2013), our hypothesis is that sakuranetin is capable of modulating vascular and lung parenchyma remodeling.

2. Methods

2.1. Plant material

Twigs of *B. retusa* DC were collected in July 2009 in Campos do Jordão/SP, Brazil and the plant material was identified by Dr. Oriana A. Fávero, and a voucher specimen has been deposited at Herbarium of Instituto de Botânica-SEMA, São Paulo/SP, Brazil.

2.2. Extraction, isolation and characterization of sakuranetin

Powdered twigs of (500 g) were extracted with MeOH at room temperature. After extraction with hexane and CH₂Cl₂, the CH₂Cl₂ phase (2.4 g) was subjected to CC over silica gel and was eluted with CH₂Cl₂ containing increasing amounts of EtOAc (up to 100%) to yield pure sakuranetin (736 mg). The ¹H and ¹³C NMR spectra, as well as the LREIMS of sakuranetin, were recorded and compared with the data of the literature (Grecco et al., 2012).

2.3. Animals and ethics statement

The experiments performed in this study were approved by the internal ethical committee of the School of Medicine of the University of São Paulo (Brazil) (Number 338/10). Male BALB/c mice aged 6–8 weeks (25–30 g) were used in the experiments, were acquired from the Universidade de São Paulo, and were maintained in our own animal facilities. The animals used in the present study received humane care in compliance with the rules of the British Pharmacological Society's Ethics Committee. They were kept on a 12 h light/dark cycle in a temperature-controlled room at 21–23 °C, with free access to water and food.

2.4. Ovalbumin sensitization protocol

Mice were randomly divided into four groups (8 animals in each group): (a) control (subjected to a saline protocol and treated with vehicle); (b) OVA (ovalbumin, subjected to the OVA sensitization and treated with vehicle); (c) OVA + SK (ovalbumin-sakuranetin, subjected to the OVA sensitization and treated with sakuranetin); (d) OVA + DX (ovalbumin-dexamethasone, subjected to the OVA sensitization and treated with dexamethasone).

They received OVA (50 µg i.p.) (GRADE IV, Sigma Aldrich, St. Louis, USA) in the presence of 6 mg of an Al(OH)₃ adjuvant (Pep-samar, Sandei-Synthelabo SA, Rio de Janeiro, Brazil) diluted in 0.2 mL of saline solution on days 1 and 14 as previously described (Toledo et al., 2013). One week after the second i.p. injection, aerosolized OVA were performed on days 22, 24, 26 and 28. For this treatment, the mice were placed in a Plexiglas box (30 cm × 15 cm × 20 cm) coupled to an ultrasonic nebulizer (US-1000; ICEL, São Paulo, Brazil) and were exposed to 1% OVA for 30 min. The control mice received the adjuvant (i.p.) and were exposed to a nebulized aerosol of saline (0.9% NaCl) at the same time points.

2.5. Sakuranetin and dexamethasone treatment

The OVA sensitized animals were treated daily with 20 mg/kg (10 µL, intranasal) of sakuranetin or 5 mg/kg i.p. of dexamethasone, starting at day 22 until the end of protocol. Sakuranetin was diluted in DMSO (Sigma Aldrich) and saline 0.9% (1:4) (Vehicle). The doses of sakuranetin and dexamethasone used in the present study were based on another study and on some articles that used a compound with similar structure (Jung et al., 2009; Toledo et al., 2013). All of the animals were anesthetized and connected to a mechanical ventilation; the lungs were removed at day 29 after 30 min of the last dose of sakuranetin, dexamethasone or vehicle treatment.

2.6. Lung morphometric analysis

To perform the histological analysis, the lungs were removed *in block*, and 5 µm thick sections of the lungs were stained with: (i) LUNA to detect the eosinophils (Luna, 1986; Lanças et al., 2006) around the pulmonary vessels and lung parenchyma; (ii) picrosirius (Direct Red 80, C.I. 35780, Sigma Aldrich) to quantify the collagen fiber in the vessel walls and lung parenchyma; and (iii) Weigert's resorcin fuchsin to quantify the elastic fibers in the vessel walls and lung parenchyma (Prado et al., 2006).

The vessel walls and lung parenchyma collagen and elastic fibers content were quantified by an observer unaware of the study groups using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany), a digital camera (Leica DFC420 Leica Microsystems, Wetzlar, Germany) and the image analyses software Image Proplus 4.5 (Media Cybernetics, Silver Spring, USA).

Five vessels and fifteen lung parenchyma representative fields at 400x magnification were evaluated for each animal. The colla-

gen and elastic fiber contents were evaluated in the area of interest using a predetermined filter. The positive area of collagen and elastic fibers represented the relationship between the quantity of collagen or elastic fibers in a specific frame and the total area of that frame (as a percentage). To ensure that we were analyzing pulmonary vessels with similar sizes and avoiding size interference in the positive area analyzed, we measured the external muscle layer perimeter of the pulmonary vessels, discarding the adventitia region that had greater edema, which could interfere with the airway area. Therefore, we noted the need for standardization in the positive area analysis. The results are expressed as the collagen or elastic fiber content adjusted by the external muscle layer perimeter in μm or by the collagen or elastic fiber of the total area of lung parenchyma (Shiang et al., 2009). In lung parenchyma, the total area of the frame was $26,842.81 \mu\text{m}^2$.

The eosinophils were evaluated using a point-counting technique (Weibel, 1963). To determine the eosinophils/unit area ($10^4 \mu\text{m}^2$) (Angeli et al., 2008; Prado et al., 2006; Toledo et al., 2013; Righetti et al., 2014), we counted the number of points of the integrating eyepiece falling on the areas around the peribronchial vessels or falling on the alveolar septum and the number of eosinophils in the same areas. All of the analyses were performed in five representative fields scattered in preparation for the peribronchial vessels, and fifteen lung parenchyma fields at 1000x magnification were examined for each animal. The results are expressed in cells per unit of area.

Using the image analyzer for the evaluation of the muscle layer thickness, we quantified the distance of the internal and outer layer of the muscle in 5 vessels per animal. The results were expressed in μm .

2.7. Evaluation of 8-PGF-2 α and NF- κB expression

The immunohistochemical staining was performed using an antibody to anti-8-epi-PGF2 α (goat polyclonal IgG, IS20, Oxford Biomedical Research, Oxford, England) and anti-NF- κB (rabbit polyclonal IgG anti-p65-NF- κB , SC109, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:10,000 and 1:400 dilutions, respectively (Angeli et al., 2008). The ABCkit Vectastain (Vector Elite PK-6105, Burlingame, USA) was used as the secondary antibody, and DAB (Sigma Aldrich) was used as the chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). We determined the 8-iso-PGF2 α stained area by image analysis as described above in five representative fields scattered in the preparation for peribronchial vessels, and fifteen lung parenchyma fields at 400x magnification were examined for each animal. The measurements were carried out at 400x in each slide, and the results are expressed as 8-isoprostane adjusted by the external muscle layer perimeter in μm or as 8-isoprostane adjusted by the total area of the lung parenchyma.

Positive NF- κB immunostained cells were evaluated using a point-counting technique, as described above in the LUNA evaluation. To determine the positive cells/unit area ($10^4 \mu\text{m}^2$) (Angeli et al., 2008; Prado et al., 2006), we counted the number of points of the integrating eyepiece falling on the areas around the peribronchial vessels or falling on the alveolar septum and the number of positive cells in this same area. All of the analyses were performed in five representative fields scattered in the preparation for the peribronchial vessels, and fifteen lung parenchyma fields at 1000x magnification were examined for each animal. The results are expressed as cells per unit of area.

2.8. Cytokine analysis

Cytokine measurements in the lung homogenate were performed using Bio-Plex in other 6 animals from each group. For this

experiment, the lungs were quickly removed and quickly frozen, and a Bio-Plex mouse Plex cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to test the samples for the presence of one growth factor in the lung homogenate. The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software version 4.0. The standard curves ranged from 32,000 to 1.95 pg/mL, as previously described (Correa-Costa et al., 2010). The results of vascular endothelial growth factor (VEGF) in the lung homogenate are expressed in pg of cytokines/pg of total protein.

2.9. Statistical analyses

The Sigma Stat software version 10 (California, USA) was used to perform statically analysis. The parametric data were analyzed by one-way ANOVA followed by Student Newman–Keuls post hoc test. The data are expressed as the means $\pm$ SE, and the graphs were drawn using Sigma Plot software version 10 (California, USA) and are shown as vertical bars with standard error. A $p < 0.05$ was considered significant.

3. Results

3.1. Sakuranetin attenuates lung inflammation in bronchial vessels and lung parenchyma

Fig. 1 shows the numbers of eosinophils around the pulmonary vessel wall (Fig. 1A) and the lung parenchyma (Fig. 1B). The OVA group presented more peribronchial vessels and lung parenchyma eosinophils compared to the control group. We noticed that the OVA+SK group had a reduced number of eosinophils in the sensitized animals (OVA group), and similar values were obtained using the corticosteroid (OVA+DX group). There was no significant difference comparing the pulmonary vessels of the control group to those of the OVA+DX and OVA+SK groups, suggesting that the treated groups had a total reduction of this parameter. In addition, sakuranetin treatment was more effective at reducing the eosinophil number in the lung parenchyma compared to the OVA+DX group, and the OVA+DX group had a higher number of eosinophils compared to that of the control group.

3.2. Sakuranetin attenuates lung remodeling

Fig. 2 shows the collagen fiber content in the vessel walls (Fig. 2A), adjusted by the external muscle layer perimeter in μm , and in the lung parenchyma (Fig. 2B). We noted that the OVA group presented an increase in collagen fiber content in the vessel walls compared to the control group. Only the animals treated with dexamethasone in the OVA+DX group presented a reduction of collagen fiber content in the vessel walls compared to that of the sensitized animals treated with vehicle. There was no significant difference between the OVA+SK group and the sensitized animals treated with vehicle. There was no significant difference between the groups treated with dexamethasone and sakuranetin in the collagen fiber content of the vessel walls.

There was no significant difference in the collagen fiber content in the lung parenchyma comparing the OVA group with the control group. Neither treatment, the OVA+SK group or the OVA+DX group, affected this parameter.

Fig. 2C shows the elastic fiber content in the vessel walls, adjusted by the external muscle layer perimeter in μm , and in the lung parenchyma (Fig. 2D). We noted that the OVA group presented an increase in elastic fiber content in the vessel walls and in the lung parenchyma compared to that of the control. Only sakuranetin (OVA+SK group) reduced the elastic fiber content both in the vessel walls and in the lung parenchyma in the sensitized animals

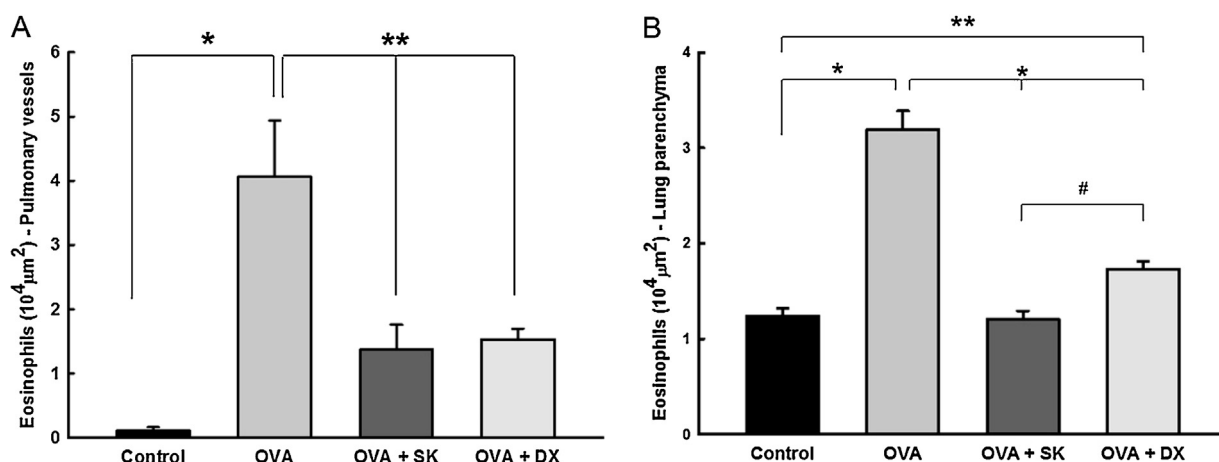


Fig. 1. Eosinophils around the pulmonary vessel wall (Fig. 1A) and lung parenchyma (Fig. 1B). The comparison among the experimental groups was evaluated by ANOVA (Significance of global comparison was $p < 0.001$ for panels A and B) followed by Student Newman–Keuls. * $p < 0.001$; ** $p < 0.01$ and # $p < 0.05$.

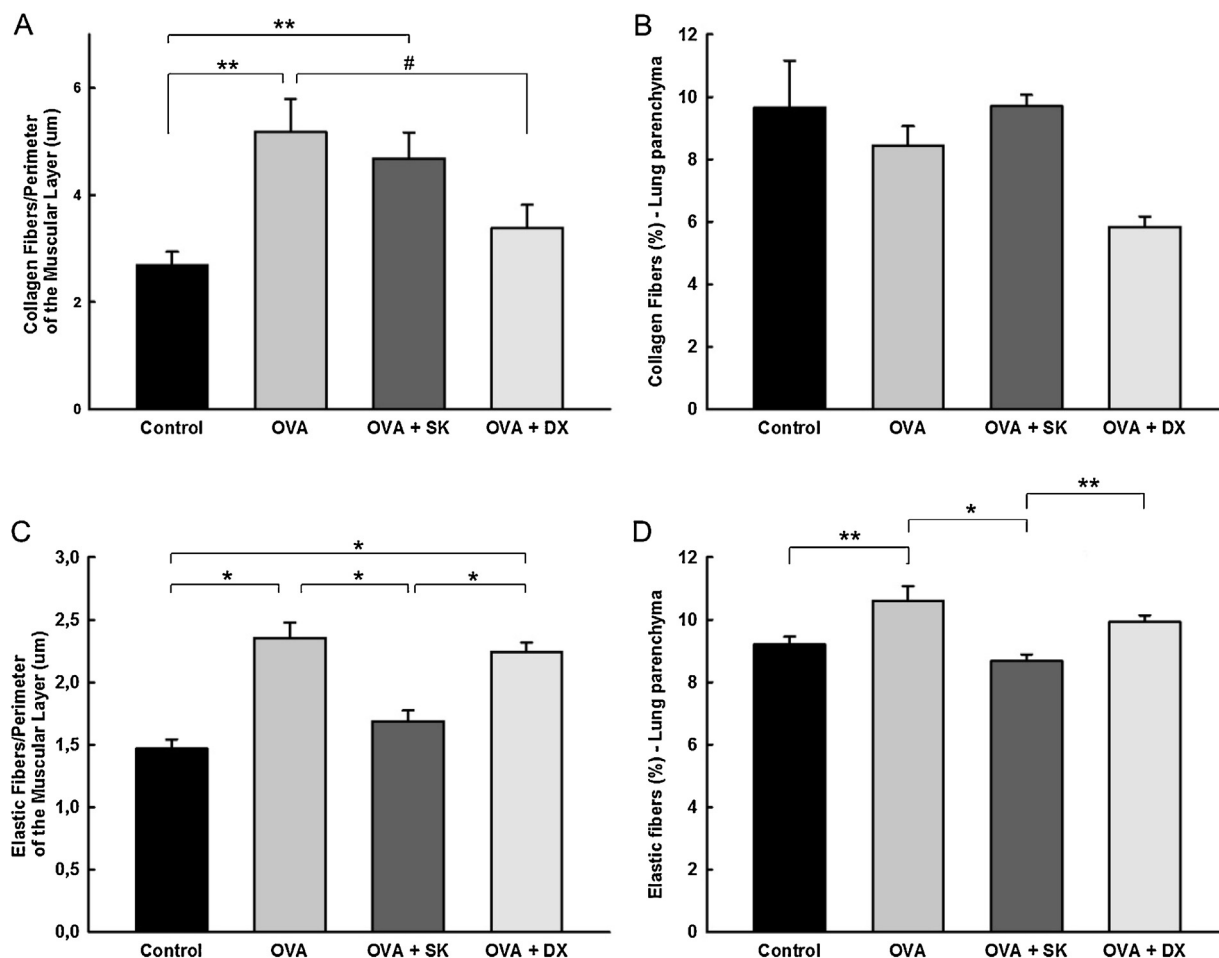


Fig. 2. Collagen and elastic fiber content in the vessel walls (Fig. 2A and C), adjusted by the external muscle layer perimeter in μm , and in the lung parenchyma (Fig. 2B and D). The comparison among the experimental groups was evaluated by ANOVA (Significance of global comparison was $p = 0.002$ for A and B and $p < 0.001$ for panels C and D) followed by Student Newman–Keuls. * $p < 0.001$; ** $p < 0.01$ and # $p < 0.05$.

compared to the OVA group. There was no significantly difference between the OVA and OVA + DX groups in elastic fiber content in the vessel walls and the lung parenchyma. Furthermore, the OVA + DX group presented a higher elastic fiber content than the OVA + SK group.

3.3. Sakuranetin reduces pulmonary oxidative stress

Fig. 3 shows the 8-iso-PGF 2α content in the pulmonary vessel wall (Fig. 3A), adjusted by the external muscle layer perimeter in μm , and in the lung parenchyma (Fig. 3B). We noted that the

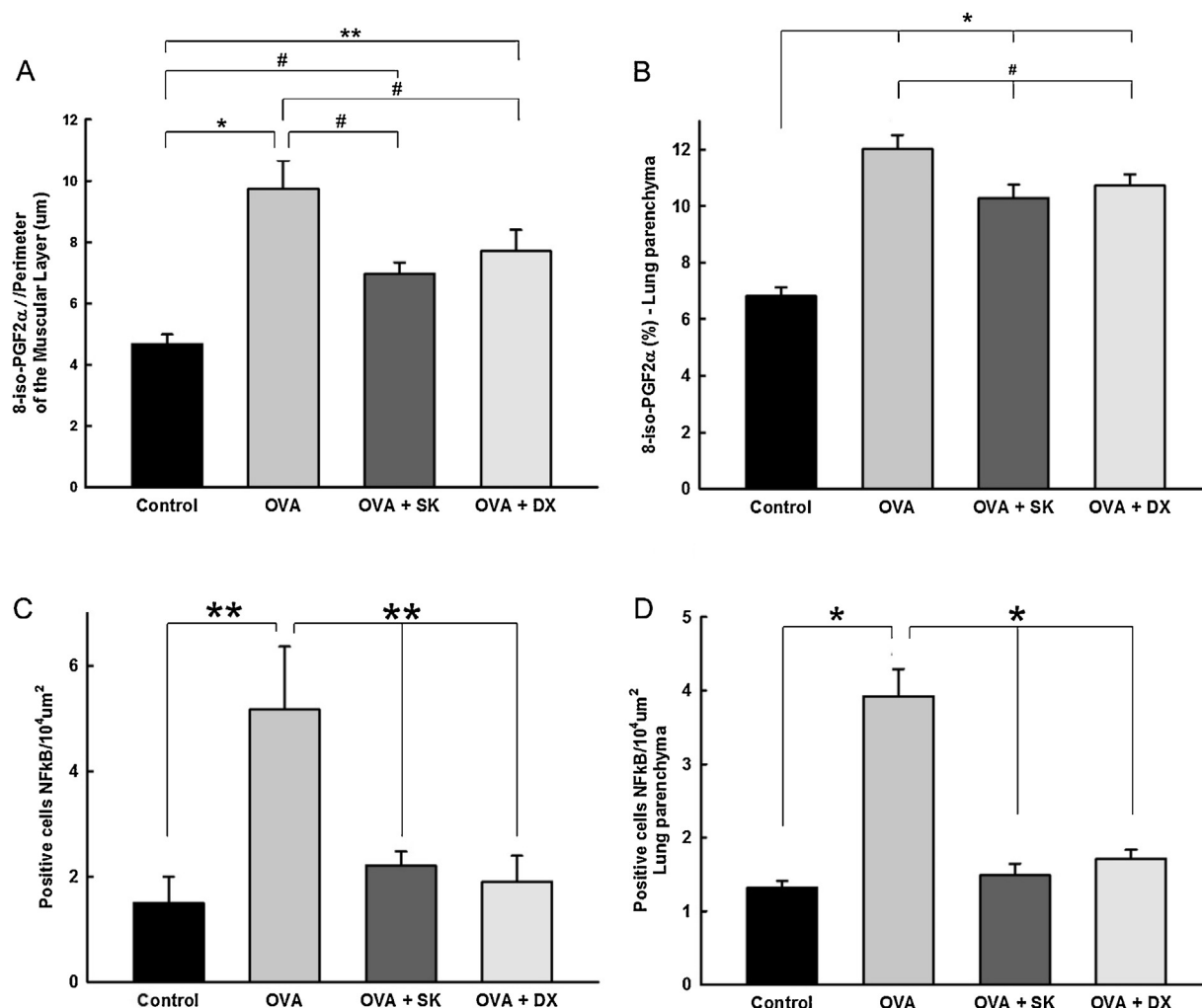


Fig. 3. 8-isoPGF2α content in the pulmonary vessel wall (Fig. 4A), adjusted by the external muscle layer perimeter in μm , and in the lung parenchyma (Fig. 4B) and positive cells no NF- κB around vessel wall (C) and in lung parenchyma (D). The comparison among the experimental groups was evaluated by ANOVA (Significance of global comparison was $p < 0.001$ for data from panels A, B and D and $p = 0.005$ for data from panel C) followed by Student Newman–Keuls. * $p < 0.001$; ** $p < 0.01$ and # $p < 0.05$.

OVA group presented an increase in 8-iso-PGF2α content in the vessel walls and in the lung parenchyma compared to the control group. Sakuranetin (OVA + SK group) reduced the 8-iso-PGF2α content in the pulmonary vessel wall and in the lung parenchyma in the sensitized animals (OVA group), and similar values were obtained with the corticosteroid (OVA + DX group). There was no significant difference comparing the sakuranetin (OVA + SK group) and corticosteroid (OVA + DX group) treatments. However, both treatments presented higher values compared to the control group.

3.4. Sakuranetin reduces NF- κB expression in the inflammatory cells

NF- κB , a transcription factor, plays a critical role in triggering and coordinating both the innate and adaptive immune response; therefore, we evaluated NF- κB expression. Fig. 3C and D shows the values of the NF- κB positive cells around the pulmonary vessel wall and in the lung parenchyma, respectively. We noted that the OVA group presented a higher quantity of NF- κB -positive cells around the pulmonary vessels and in the lung parenchyma compared to that of the control group. In addition, both treatments, sakuranetin (OVA-SK group) and dexamethasone (OVA-DX group), reduced the NF- κB -positive cells around the peribronchial vessels and the lung parenchyma compared to the OVA group. There was no

significant difference comparing the sakuranetin and corticosteroid treatments.

3.5. Sakuranetin reduces the smooth muscle layer thickness of the peribronchial vessels

Fig. 4A shows the smooth muscular layer thickness of the peribronchial vessels. We noted that the OVA group presented an increased smooth muscular layer thickness in the pulmonary vessels compared to that of the control group. Sakuranetin (OVA + SK group) reduced the smooth muscular layer thickness of the pulmonary vessels in the sensitized animals (OVA group), and similar values were obtained with the corticosteroid (OVA + DX group). There was no significant difference comparing the sakuranetin and corticosteroid treatments. However, the sakuranetin treatment (OVA + SK group) presented higher values compared to those of the control group.

3.6. Sakuranetin reduces VEGF

We noted that the OVA group had increased VEGF levels in the lung homogenate compared to those of the control (Fig. 4B). Sakuranetin (OVA + SK group) reduced the levels of VEGF in the lung homogenate in the sensitized animals (OVA group), and similar values were obtained with the corticosteroid (OVA + DX group). There

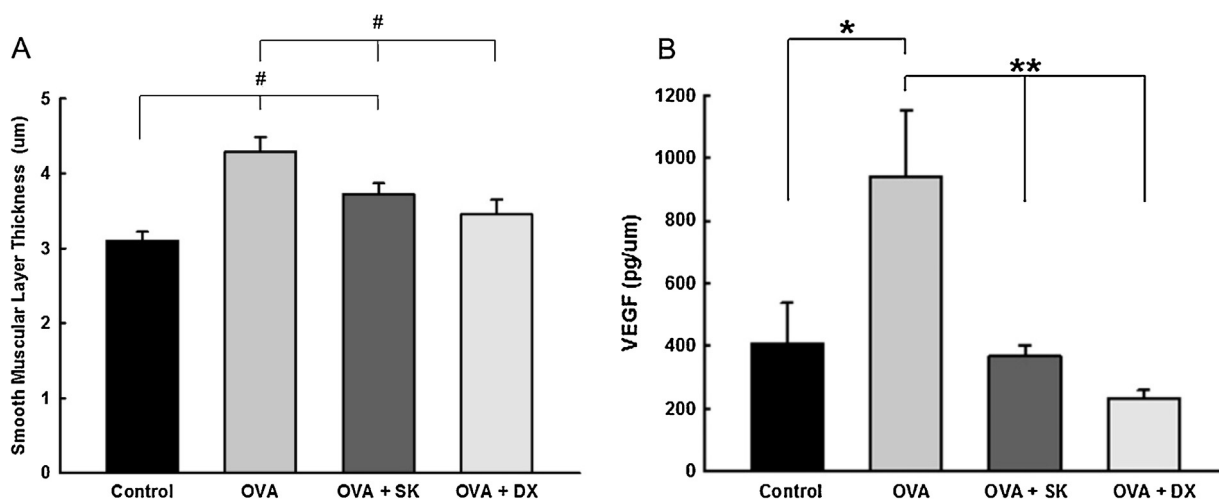


Fig. 4. Smooth muscular layer thickness of the peribronchial vessels and VEGF levels detected in lung homogenate. The comparison among the experimental groups was evaluated by ANOVA (Significance of global comparison was $p < 0.05$ for data from panels A and $p = 0.002$ for panel B) followed by Student Newman–Keuls. $^{**}p < 0.01$ and $^{\#}p < 0.05$.

was no significant difference comparing the sakuranetin treatment (OVA + SK group) and corticosteroid treatment (OVA + DX group).

3.7. Qualitative analysis

Representative photomicrographs of the oxidative stress and remodeling in the pulmonary vessel walls and the lung parenchyma (Fig. 5) show cells stained for 8-isoPGF2 α , collagen and elastic fiber content.

The OVA group had high levels of 8-isoPGF2 α , collagen and elastic content, suggesting lung inflammation, remodeling and an oxidative stress process. Both treatments with OVA + SK reduced oxidative stress and the elastic fiber content in the pulmonary vessels and in the lung parenchyma, as observed in the representative photomicrographs.

4. Discussion

In the present study, we showed that treatment with sakuranetin reduces the content of elastic fibers in the peribronchial vessels and in the lung parenchyma. In addition to the contents of VEGF in the lung, these effects are associated with reduced eosinophilic inflammation, an increased expression of the transcription factor NF- κ B and the activation of oxidative stress in the peribronchial vessels and in the lung parenchyma.

The most important pathogenic factor of asthma is inflammation, and this is the result of the complex interactions of inflammatory cells, mediators and structural airway cells. It is present in patients with recent onset asthma, in those with mild forms of the disease and even among asymptomatic patients. The inflammatory response after exposure to an allergen has special features that include eosinophilic infiltration; the release of pharmacologically active mediators from mast cells and basophils; growth factors, such as VEGF; the interstitial injury of the airway wall; and the activation of Th2 lymphocytes that produce cytokines, such as IL-4, IL-5, IL-6 and IL-13, among other things, that are responsible for the initiation and maintenance of inflammation (GINA, 2014).

Several experimental models are used to study asthma (Takeda and Gelfand, 2009; Kips et al., 2003). In this study, we chose to use a mouse model with Balb/c lineage. The sensitization protocol used was based on previous studies from our laboratory, which demonstrated that this model has bronchial hyperresponsiveness

and eosinophilic infiltration, as well as increases in specific IgE antibody and nonspecific IgG (Arantes-Costa et al., 2008). Toledo et al. (2013), confirming these findings, showed that the Balb/c mice exhibit eosinophilia, hyperresponsiveness, remodeling, and increased oxidative stress and NF- κ B in their airways. In the present study, we expanded these findings and observed vascular and pulmonary parenchymal changes.

Animals sensitized with ovalbumin and treated with vehicle showed eosinophilia in the peribronchial vessels and in the lung parenchyma, showing the presence of inflammation in more than just the airways. Moreover, the OVA group showed an increase in the thickness of the muscle layer of the vessels, in oxidative stress, in the amount of vascular elastic fibers in the distal lung tissue, and in the amount of perivascular collagen fibers compared to those of the control, confirming their presence and suggesting vascular and distal lung tissue remodeling in this model. In addition, there was an increase in VEGF in the lung.

It is known that structural changes that occur are mainly present in the asthma airway, and thus, most studies of asthma pulmonary focus on only this region. However, there is a correlation between tissue remodeling with changes in the pulmonary parenchyma in asthma because it may affect the entire bronchial wall, including the mucosal vascular component given that the peripheral lung tissue responds to bronchoconstrictor stimuli obstructing the flow air (Dolhnikoff et al., 1998; Yanai et al., 1992; Ohruai et al., 1992; Hamid, 2012).

Secondary to the action of the inflammatory mediators and growth factors, there is great bronchial vasodilation at the capillary level and in the post-capillary veins of asthmatics. There is an even greater number of vessels, and these are of a larger gauge (Mauad et al., 2000). The final result of this condition is the presence of edema, and it is assigned to a state of “leakage” of the bronchial microcirculation. This increased vasculature, with an altered state of permeability, has implications for the mechanism of bronchial remodeling (Mauad et al., 2000). Thus, vascular disorders may contribute to obstruction of flow to the airways in asthma (Chetta et al., 2007).

Zanini et al. (2010) and Detoraki et al. (2010) showed that microvascular abnormalities in asthma may contribute to an increase in the wall thickness of the airways and a reduction of the bronchial lumen, which may be associated with the disease progression area. Our results confirm the importance of structural and inflammatory changes in the vessels in this model of asthma.

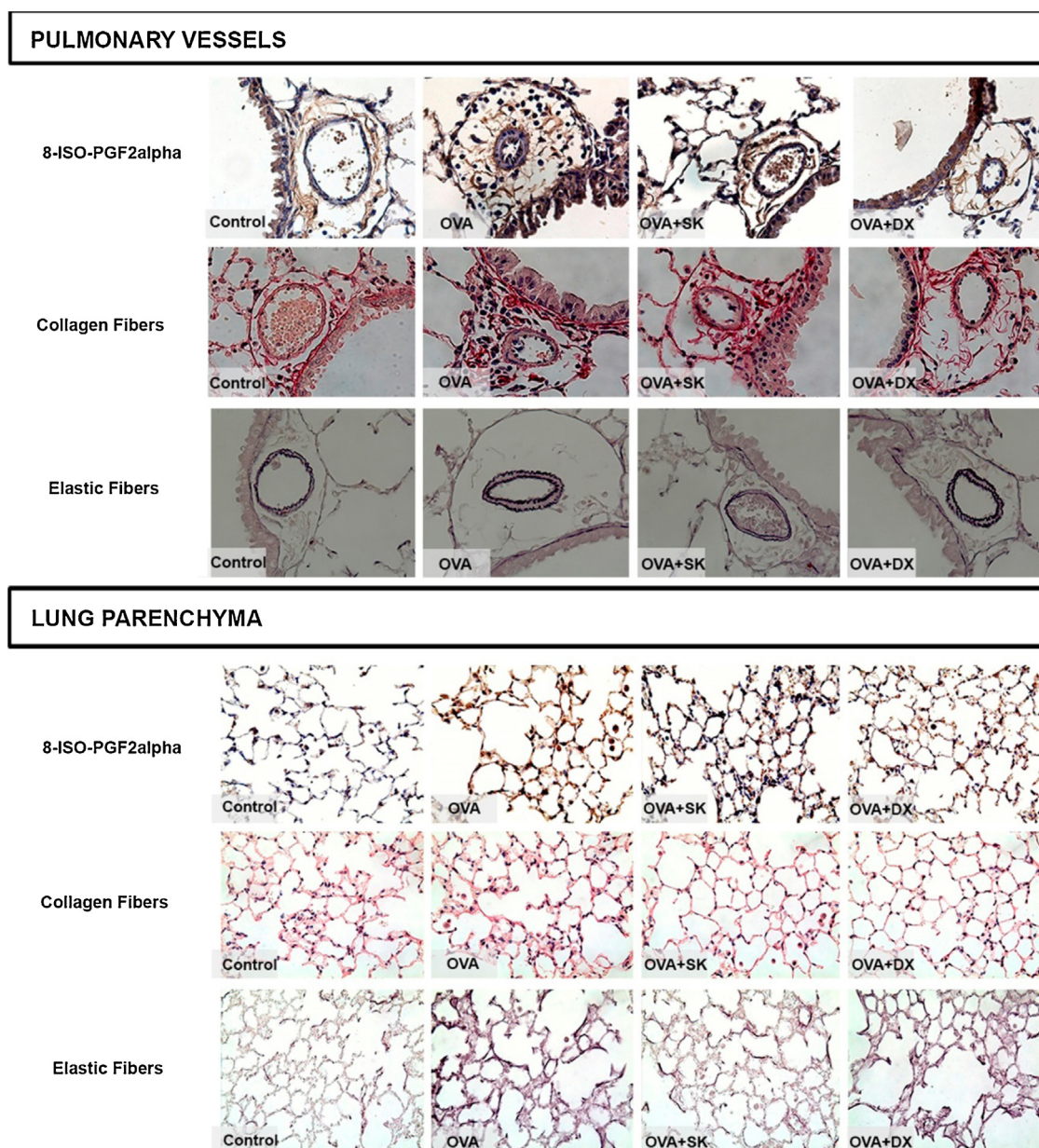


Fig. 5. Representative photomicrographs of the oxidative stress and remodeling in the pulmonary vessel walls and in the lung parenchyma.

Several studies of bronchial vascular remodeling in asthma showed significant correlations between morphological and biological data (number of vessels, vascular area, and expression of angiogenic factors) and parameters of lung function and airway hyperresponsiveness (Zanini et al., 2010). Regarding the lung tissue, Spears et al. (2006) demonstrated that it is involved in the late asthmatic response in guinea pigs with chronic allergic pulmonary inflammation, which correlates with eosinophil recruitment and extracellular matrix remodeling. Our results confirm the importance of structural and inflammatory changes in the lung tissue in this model of asthma.

Corroborating these results, Simões et al. (2005) demonstrated the presence of distal lung inflammation with the involvement of the peripheral lung parenchyma and the peripheral airways in asthmatics. Kraft et al. (1996) demonstrated that macrophages and eosinophils accumulate to a greater extent in the peripheral lung parenchyma, and this contributes more to the variation in pulmonary function compared with the most proximal tissue

inflammation. These findings suggest that the distal lung may be a target of the anti-inflammatory treatment, which contributes to better control of the disease.

The demand for herbal treatments, plants used in folk medicine, which may or may not be associated with conventional treatments, has been increasing in recent years. Among the various plant species used in folk medicine are those belonging to the family Asteraceae *Baccharis* that are used to control or treat various diseases (Verdi et al., 2005). The species *Baccharis retusa* mainly contains the derivative flavonoid 5,4'-dihydroxy-7-methoxyflavanone (sakuranetin), a polyphenol derivative produced by the metabolism of the plant.

Although the mechanisms of action involved in the anti-inflammatory effects of sakuranetin were also an aim of the investigation, it was demonstrated that sakuranetin inhibits ERK1/2 phosphorylation, without effecting p38 and JNK phosphorylation. It was also demonstrated that sakuranetin increases the in vitro activity of tyrosinase and up-regulates tyrosinase-related

protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2). Moreover, sakuranetin inhibits the phosphorylation of AKT at threonine 308 and serine 473. In a previous study, our group demonstrated that sakuranetin inhibits the protein expression of the p65 subunit of NF- κ B in the lung (Toledo et al., 2013; Taguchi et al., 2015). Furthermore, the expression of some pro-inflammatory genes, such as lipoxygenase, NOS and cyclooxygenase are reduced by flavonoids (Siebenlist et al., 1994).

Is important to emphasize that sakuranetin is a potent and known anti-oxidant, and because oxidative stress could also perpetuate the inflammatory response, it should be considered that the anti-inflammatory effects of sakuranetin in this model could be due to an anti-oxidant effect. The antioxidant effects of flavonoids are related to the presence of an impaired electron in the aromatic ring as well as the capacity of hydrogen radical donation from the phenol groups. In addition, flavonoids reduce oxidative stress by stimulating glutathione-S-transferase (Lago et al., 2014).

The study of new pharmacological compounds with goals to improve lung function and/or to reduce the infiltration of eosinophils is desirable and necessary in cases of the treatment of patients with atopic/allergic diseases, syndromes and other hyper eosinophilic-related diseases. Therefore, the development of new classes of drugs is required to inhibit the activation of eosinophils and other therapeutic targets, which are suppressed by corticosteroids.

Some previous studies have reported that flavonoids exert antiallergic effects in murine models of asthma (Das et al., 2003; Funaguchi et al., 2007; Rogerio et al., 2007; Wu et al., 2006). However, the pharmacology composition of these compounds differs from one type to another. Rogerio et al. (2010) showed that quercetin, a flavonoid, has an anti-inflammatory effect in a mouse model of asthma, reducing the number of leukocytes and Th2 cytokines. However, these studies evaluated only inflammation, without observing the effect on pulmonary and vascular remodeling. To the best of our knowledge, this is the first study to evaluate the effect of this flavonoid in reducing the inflammation of vascular tissue and the lung parenchyma.

Increased levels of eosinophils during inflammation cause a release of mediators that contribute to the pathological changes of asthma (Wu et al., 2006). Still, eosinophils facilitate fibroblast proliferation, collagen synthesis and the maturation of myofibroblasts, leading to the occlusion of the bronchial lumen (Kay et al., 2004), which contributes to collagen deposition and increased alveolar damage (Mahmoud, 2012). Treatment with sakuranetin reduced the vascular wall and eosinophils in the lung parenchyma similarly to treatment with corticosteroids.

In relation to vascular remodeling and the lung parenchyma, the sakuranetin treated animals showed a reduction in the amount of elastic fibers compared to those of the sensitized animals. This effect was not observed in the animals treated with glucocorticoid. In the activation of matrix metalloproteinases (MMPs), there is an excessive deposition of collagen. Another hypothesis is that sakuranetin would not have affected the levels of the cytokine TGF- β , which is known to stimulate the expression and activation of MMPs. However, treatments with corticosteroids or sakuranetin reduced the thickness of the muscular layer of the pulmonary vessels. Together, these data suggest that sakuranetin, as well as the corticosteroids, acts in vascular remodeling and in distal tissue.

Some studies corroborate our findings and show changes in the walls of the airways, such as bronchial remodeling and vasodilation with increased vascularization (Li and Wilson, 1997; Velden et al., 2012). This may be associated with a greater number of vessels, with vessels with diameters of greater thickness, and with the presence of edema.

Part of the vascular remodeling observed was that sakuranetin also reduced the thickness of the vascular wall in the sensitized and

treated animals, which again shows a protective effect on vascular remodeling. It is known that the vascular endothelial growth factor (VEGF), a mediator derived primarily from endothelial cells but also produced by inflammatory cells, plays a fundamental role in the vascular remodeling and angiogenesis that occurs in asthma. The modulation of angiogenesis is regarded as an extremely important therapeutic strategy for human health. In recent years a growing number of studies describe the role of VEGF in the course of chronic inflammatory respiratory diseases (asthma and COPD). VEGF is implicated in the pathogenesis of allergic asthma by increasing inflammation, vascular permeability and mucus secretion (Chetta et al., 2007; Mojzis et al., 2008). Recently, studies showed that VEGF is significantly associated with no acceleration of airway remodeling, but can also influence the severity of asthma (Gomułka and Liebhart, 2009; Lee et al., 2011).

In addition, other studies show that VEGF plays an important role in Th2 inflammation because, in addition to stimulating angiogenesis and edema, VEGF induces eosinophilic inflammation, mucus metaplasia, subepithelial fibrosis, myocyte hyperplasia, the activation of dendritic cells, and airway hyperresponsiveness via IL-13-dependent and independent mechanisms (Lee et al., 2011).

Various bioactive plant compounds have recently been tested for their anti-angiogenic potential. Among the most commonly studied are plant polyphenols that inhibit angiogenesis and metastasis by regulating multiple signaling pathways, such as VEGF expression, matrix metalloproteinases (MMPs), and EGFR, thus causing strong antiangiogenic effects.

To evaluate the possible mechanisms interfering with the effects of the flavonoid in reducing remodeling, we quantified the oxidative stress in the vessels and in the lung parenchyma and noted that treatment with corticosteroids or sakuranetin reduced oxidative stress. This result is explained by the study of Toledo et al. (2013), which used the same experimental model as this study to understand the effect of sakuranetin inflammation. The authors observed a reduction in Th2 cytokines (RANTES, IL-5 and eotaxin) without the interference of anti-inflammatory cytokines, such as IL-10 and the Th1/Th2 balance (no effect of sakuranetin was observed on TNF- α , IFN- γ or GM-CSF). Therefore, treatment with sakuranetin reduced the pro-inflammatory cytokines from Th2 cells, the NF- κ B expression and oxidative stress without affecting the Th1 cytokines.

Allergic asthma is widely recognized as an inflammatory disorder, and recent studies have implicated the transcription factor NF- κ B as an important modulator of inflammation in the pathogenesis of lung disease, which plays a critical role in triggering and coordinating both the innate and adaptive immune response (Ather et al., 2011). We performed an immunohistochemistry study, and in fact, a reduced expression of this transcription factor was observed in the airways and the pulmonary vessel peribronchial tissue, which is a mechanism by which sakuranetin controls inflammation, corroborating the results found by Zhou et al. (2011). Various flavonoids found in *Capparis spinosa*, including sakuranetin, are involved in the *in vitro* inhibition of NF- κ B activation (Zhou et al., 2011).

We also note that, when comparing our results with those already published in previous studies (Toledo et al., 2013), it appears that treatment with sakuranetin was actually more effective in the local airways where the histopathological changes are more relevant in this model. It is also important to note that Toledo et al. (2013) showed the important effects of sakuranetin in reducing airway resistance, which mainly reflects alterations in the large airways. Moreover, sakuranetin also reduced collagen and elastic fiber deposition in the large airways. Even then, however, the effects on the reduction of vascular disorders and lung tissue were significant.

The results described above are of great clinical relevance because the effects observed in the group treated with sakuranetin

were of a similar magnitude or even greater than those obtained in the group treated with corticosteroids. Therefore, our data suggest that the flavonoid 5,4'-dihydroxy-7-methoxyflavanone (sakuranetin) possesses therapeutic potential in the control of inflammation present in bronchial asthma both in the pulmonary vessels and in the lung parenchyma.

5. Conclusion

Treatment with sakuranetin reduced eosinophilic inflammation and elastic fibers in the lung parenchyma and also worked in vascular remodeling. Thus, our results showed that treatment with sakuranetin reduced vascular disorders and the pulmonary parenchyma in this experimental model of bronchial asthma, suggesting that anti-inflammatory and antioxidant activity contribute to the effects of sakuranetin in reducing the activation of NF- κ B, VEGF and oxidative stress.

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