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Capillarisin exerts antiasthmatic activity in neonatal rats via modulating the matrix remodeling

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Abstract: The use of phytochemical plays a major role in recent therapeutic regimens. Amongst, Capillarisin (CPS), an active chemical constituent of *Artemisia capillaris* was found to exert anti-inflammatory and antioxidant properties. However, the protective role of CPS has not been identified against neonatal asthma. Hence, in the present study, Wistar rats were used consisting of four groups such as control, asthma-induced, CPS-pretreated asthma animals, and CPS control. At the end of the experimental period, histology of the lungs, inflammatory cell counts in bronchoalveolar lavage fluid (BALF), inflammatory markers such as interleukin (IL) -6, IL-5, IL-4, and IL-13 were measured. Results demonstrated a significant restoration in alveolar thickening and reduced goblet cell hyperplasia with suppressed inflammatory cells. Moreover, a significant reduction in leukocyte infiltration in BALF lessened hyper responsiveness, and serum IgE levels of CPS treated group. Furthermore, the CPS administration alleviated the expression levels of IL-6, IL-17, IL-4 and IL-13 compared to the asthma-induced group. To an extent, the study elicited the extra cellular matrix protein expression in the asthma-induced animals, and the results demonstrated a profound reduction in the fibrotic markers was evidenced in CPS treated animals. Thus, the results of the present investigation propose that capillarisin can be a new medicine target for the treatment of asthma-mediated complications.

Keywords: Capillarisin, antiasthmatic activity, neonatal rats, matrix remodeling

INTRODUCTION

Asthma is a major chronic inflammatory disease of the lungs and affects the worldwide populace of all ages. It affects the airways due to infiltration of eosinophils, airway hyperresponsiveness and remodeling of the airway structure (Grzela *et al*., 2016) due to the infection. Allergy due to air pollution from pollen (Davies, 2014, Grzela *et al*., 2016) and dust deposition by wind (Sheats *et al*., 2019) (Weber *et al*., 2015) (Esty *et al*., 2019, Kc *et al*., 2018) due to urbanization, dysregulated burning of farm waste and municipal waste (Ramakreshnan *et al*., 2018) and many other factors are causatives of asthma. Asthma manifests itself in various forms such as airway inflammation, hypertrophy, hyperplasia of airway smooth muscle cells and airflow obstruction by blockage of the airway with mucus from epithelial goblet cells to cause wheeze, cough, and discomfort in the chest and increase the airway wall thickness (Carroll *et al*., 1996, James *et al*., 2012, James *et al*., 2002). The inflammation in the airway passage due to asthma causes tissue injury and that develops more structural alterations that are known to be airway remodeling (Castro-Rodriguez *et al*., 2019). Inflamed airway would necessarily mean structural alterations in the airway epithelium, sub-epithelium, airway smooth muscle, and vasculature. Thickening of airway increases in such cases and blocks the air passage denotes the severity of this prolonged inflammatory disease. Migration of smooth muscle cells towards the lung epithelium and increase in muscle mass in airway tissues are hallmarks of the structural remodeling in asthmatic airway inflammation (An *et al*., 2007). Epithelial cells that protect the airway secrete various inflammatory cytokines (Bartemes and Kita, 2012) and growth factors (Zhang *et al*., 1999) that mediate the remodeling by affecting the extracellular matrix production and fibroblast proliferation.

Rapidly rising cases of asthma in developing countries due to the usage of outdated technologies, rising human and vehicular population and lifestyle. Specific drugs are prescribed in the forms of corticosteroid inhalers, beta-2-agonists (Silva and Jacinto, 2016) and bronchodilators which would reduce the bronchial congestion by mucus and reduces the inflammation. Such medicines may give a temporary cure to the symptoms (Johnston and Edwards, 2009), but asthma cannot be cured completely. Hence, prolonged usage of such medicines in chronic patients has deeply influenced the economy of the poor who are more vulnerable to asthma. Children who developed asthma at their young age have their immunity compromised (Ulrich and Palacios, 2019) and are frequently affected whenever there is a sudden change in their environment. Administering medicines self to them would be a challenge (Bisgaard *et al*., 2006) and such medicines would have an impact on their growth in the long term. Hence, there is always an alluring note for the drug molecules that exhibit lessened side effects with effective curing ability. In this regard, the use of photochemical has been in the attempt of many researchers for the application of a wide variety of illnesses.

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Among the various herbs in medical practice, the traditional herb Artemisia capillaries, whose main bioactive compound, Capillarisin (CPS) has been used in the traditional medicines of Asia for centuries. It has been used in the treatment of liver disorders, diabetes and recently against cancer (Chu *et al*., 1999). The antioxidative (Chu *et al*., 1999), (Kim *et al*., 2017), anti-inflammatory (Han *et al*., 2013) and analgesic potential of CPS makes it an attractive molecule to be used in pharmacological applications. Many animal and cellular models utilizing CPS have been worked upon and found that it is effective against pro-inflammatory mediators such as TNF-α, IL-6, and IL-1β (Han *et al*., 2013). Since it has been already used against inflammatory pain models and the pharmacological application utilizing its anti-inflammatory properties in asthmatic models have not been so far researched. Hence, in the present study, we have attempted the experimental asthmatic animal model to evaluate the effect of CPS. Since the course of asthma proceeds with the airway remodeling earlier, we have introduced the inflammation in the lungs in our animal models by intra-peritoneal administration of OVA. Our hypothesis is that CPS would exert an anti-asthmatic activity in the animals by directly reducing the inflammation and airway remodeling by decreasing the expression of inflammatory cytokines and growth factors that aid in the airway remodeling.

MATERIALS AND METHODS

Asthma experimental animal model

For the present study, Wistar strain rat pups were used. The prior permission was obtained from the Institutional Animal Care and Use Committee, China, for the procedures implemented in the present study. These animals were fed with reverse osmosis water and commercial rat chow. The animals were housed in pathogen-free cages kept at the temperature of 20–25°C with 50–70% relative humidity. Wistar rats pups of 10 days old were used in the present study were divided into 4 groups with 12 animals in each group as follows. Group 1: Vehicle-treated, Group 2: asthmatic group (Ova administered), Group 3: asthma rats treated with Capillarisin (50mg/kg, Oral administration), Group 4: Capillarisin control. Capillarisin was purchased from Wako (Osaka, Japan). The protocol for the induction of asthma in rat pups was obtained from the previously published reports with little modifications (Hao *et al*., 2019). Briefly, the pups were sensitized with intraperitoneal administration of OVA (20 µg) on day 1 and 7th and from the 14th until 21st days along with 30 min aerosol exposure of 1% OVA from 14th day onwards. At the end of the experimental period, the animals were killed, and the lung tissues were collected, a portion of tissue was fixed in 10 % formalin solution, embedded in paraffin wax and 10µm thick sections were made stained with hematoxylin and eosin (H&E). In addition, the assessment of airway Hyper-responsiveness was estimated as per the previous publications (Hwang *et al*., 2017).

Analysis of broncho-alveolar lavage fluid (BALF)

The assessment of inflammatory cells in the BALF and serum was done by collecting the cells in the fluid, stained with Wright's staining and using a hemocytometer, the differential cell count was calculated in the control and experimental groups. In addition, IgE levels, and nitrotyrosine was estimated using commercial assay kits as per the company's guidelines (Abcam Inc, USA).

Estimation of inflammatory cytokines

The assessment of pro-inflammatory and anti-inflammatory cytokines such as IL-6, IL-4, IL-10, IL-5, and IL-17, IL-13 in the serum samples and BALF samples was estimated using commercial ELISA kits as per the manufacturer's instruction (Fn test biotech, China).

Reverse Transcription-PCR

In order to elucidate the mRNA expression of genes related to the extra cellular matrix, the total RNA was isolated from the lung tissues using TRIzol reagent. Initially, the tissues were homogenized using TRIzol, and to the homogenate, chloroform was added and centrifuged, the upper aqueous layer was collected, and an equal amount of isopropanol was added and centrifuged. To the precipitate, ethanol was added and centrifuged. The pelleted RNA was washed and quantified. About 20 µl total RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit. The real-time RT-PCR was done for specific genes using SYBR® Green PCR Kit and the gene-specific primers used in the present investigation were listed in table 1. The Ct values were used, and the gene expressions were determined by the comparative Ct method (ΔΔCT). The fold increase of the gene of interest was analyzed using GAPDH as control- house-keeping gene.

STATISTICAL ANALYSIS

Statistical significance was assessed using Graph pad prism software (Version 5.0). The statistical examination was performed using student t-test and the differences between groups with a p-value less than 0.05 were considered statistically significant.

RESULTS

Fig. 1 represents the hematoxylin and eosin staining of lung tissues of control and experimental groups. The

histological analysis demonstrated asthma induced rats exhibited the characteristic accumulation of smooth muscle mass with mucous gland hypertrophy compared to control. Meanwhile, rats co-administered with CPS displayed a significant reduction in smooth muscle matrix accumulation (fig. 1A). In addition, the analysis of the infiltration of eosinophils into BALF exhibited less than 1.0 % of total cells in normal control rats, while the infiltration was very prominent and reached to the levels >60% in asthmatic rats. Further, a higher level of IgE, nitrotyrosine and PCO levels was found in asthma-induced rats. However, rats have the CPS treatment showed a significant reduction in the asthmatic symptoms (fig. 1).

Furthermore, the analysis of airway functioning of control and experimental of rats was elucidated, and the results are presented the fig. 2. Rats induced with asthma demonstrated an increase in respiratory system resistance (Rrs), elastance (Ers), Newtonian resistance (Rn), and tissue elastance (H). However, these airway functioning parameters were found to be restored in CPS drug-treated rats compared to asthmatic animals (fig. 2).

Additionally, the analysis of the cytokines levels in control and experimental of rats is presented in fig. 3. The results displayed that the asthma rats established a significant upsurge in the levels of cytokines such as IL-6 ($p<0.01$), IL-4 ($p<0.001$), IL-5 ($p<0.001$), IL-17 ($p<0.01$), IL-13 ($p<0.01$) and reduced IL-10 ($p<0.01$) compared to control. While these inflammatory cytokines were reduced in CPS treatment with elevated levels of IL-10, demonstrate the protective effect of CPS is also through the modulation of inflammatory molecules (fig. 3).

To substantiate the role of CPS on the modulation of matrix proteins, the mRNA levels of fibrotic genes were elucidated in the control and experimental of rats, and the results were presented in fig. 4. The results demonstrated that a profound increase ($p<0.01$) in the mRNA transcript expression of MMP-2 (3-fold), MMP-9 (4.2-fold), Fibronectin (2.9-fold), VEGF (3.1-fold), CTGF (3.6-fold), TGF-β (3.8-fold) in rats with asthma compared to vehicle-treated controls. However, the increased levels of these matrix proteins were attenuated in CPS treatment indicate the protective effect of CPS against neonatal asthma is through the modulation of matrix-associated proteins (fig. 4).

DISCUSSION

Bronchial asthma is caused due to chronic allergic response to various allergens (Jeffery, 2001) and causes inflammation (Kalita *et al*., 2013) in the airway that results in tissue destruction to structural changes known to be airway remodeling (Yang *et al*., 2013). Immune cells, as a result of inflammation, like T-helper cells, eosinophils and mast cells infiltrate into the airway and interact with the fibroblast, smooth muscle cells, and lung epithelial cells (Sethi *et al*., 2012, Yang *et al*., 2013). They secrete inflammatory cytokines, chemokines, metalloproteases, metabolites and growth factors (Kemi *et al*., 2006, Yang *et al*., 2013) that provide conducive environment for the structural alterations into airway remodeling which occurs by means of loss in epithelial integrity, basement membrane thickening (Wilson and Li, 1997), fibrosis in the sub-epithelial tissues (Churg *et al*., 2009, Kranenburg *et al*., 2006), enlargement of goblet cells, increase in the mass of smooth muscle cells and loss of cartilage integrity.

The number of smooth muscle cells of the airway improved in the asthmatic rats as indicated in our histological experiments which are in concordance with the pathogenesis of asthma (An *et al*., 2007) in the rats. The duration (Bai *et al*., 2000) with which these animals were affected is clearly reflected in the increased amount of smooth muscle cells (Fixman *et al*., 2007, Jeffery, 2004), cell volume in tissue and mucous gland. The contraction in the smooth muscle cells would cause broncho-constriction (Gosens and Grainge, 2015, Grainge *et al*., 2011) which relates to airway remodeling and hence smooth muscle cells play a major role in the pathogenesis of asthma and hence airway remodeling in our experimental asthmatic rats.

Moreover, asthmatic inflammation of the airways, eosinophilic infiltration is the main feature of the process (Kerzel *et al*., 2009). In our OVA-induced asthma model, the asthmatic animals exposed increased inflammation of the airways due to increased infiltration of the eosinophils observed in the BALF that is characteristic of the OVA-induced asthmatic model (Yu and Chen, 2018). Inflammatory cells generate ROS as a result of inflammation (Sahiner *et al*., 2011) and the increased eosinophils in asthma produce superoxide anions that react with nitric oxide (Barnes and Kharitonov, 1996) in turn induces peroxynitrite anions. The formation of peroxynitrite has downstream effects on the protein in their oxidation of sulphydryl residues of proteins (Misso and Thompson, 2005). A reduction of Sulphydryl groups in the proteins of asthmatic rats (Nadeem *et al*., 2003) and a simultaneous increase in the protein carbonyl was observed in CPS treated animals. Further, the oxidant protein carbonyls production stimulates the addition of highly reactive nitro group to tyrosine to produce stable nitrotyrosine (Ischiropoulos, Zhu *et al*., 1992) for which the airway epithelium and lung parenchyma are highly reactive to it (Kaminsky *et al*., 1999, Saleh *et al*., 1998) and gets reduced with steroid inhalation treatment. The presence of nitrotyrosine and its immunoreactivity to the inflammatory cells indicates the formation of reactive nitrogen species in the lung (Kaminsky *et al*., 1999). Such a highly reactive molecule (Beckman *et al*., 1990) in our

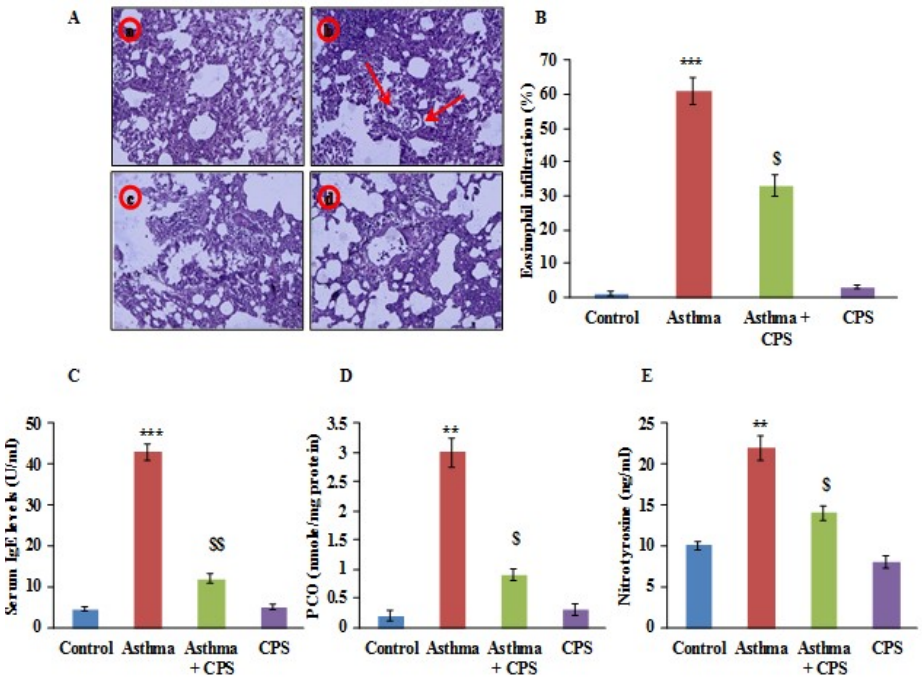

Fig. 1: A) The lung tissue histology; B-E) Represents the eosinophils, IgE, nitrotyrosine and protein carbonyl levels of BALF in control and experimental group of rats. The experimental details were given in the methodology section. Values are expressed as mean ± S.E (n = 8). Statistical significance expressed as **p<0.01, ***p<0.001 compared to vehicle-treated controls, $p<0.05, $$p<0.01 CPS compared to asthma rats; ns denotes non-significant.

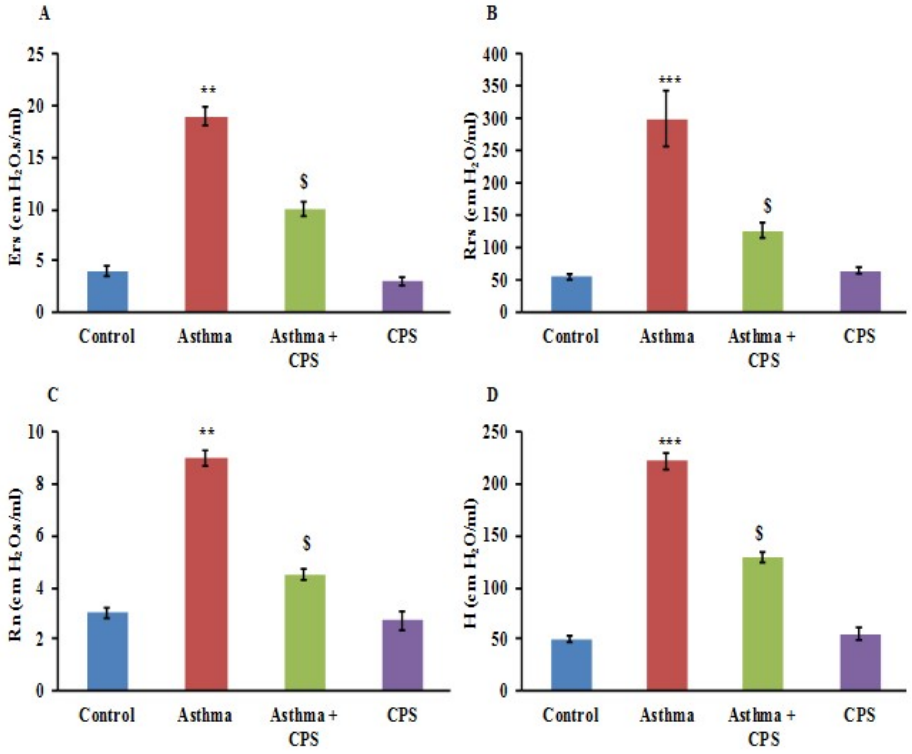

Fig. 2: A-D represents the respiratory system resistance (Rrs), elastance (Ers), Newtonian resistance (Rn), and tissue elastance (H) in the control and experimental group of rats. Values are expressed as mean ± S.E (n = 8). Statistical significance expressed as **p<0.01, ***p<0.001 compared to vehicle-treated controls, $p<0.05 CPS compared to asthma rats; ns denotes non-significant.

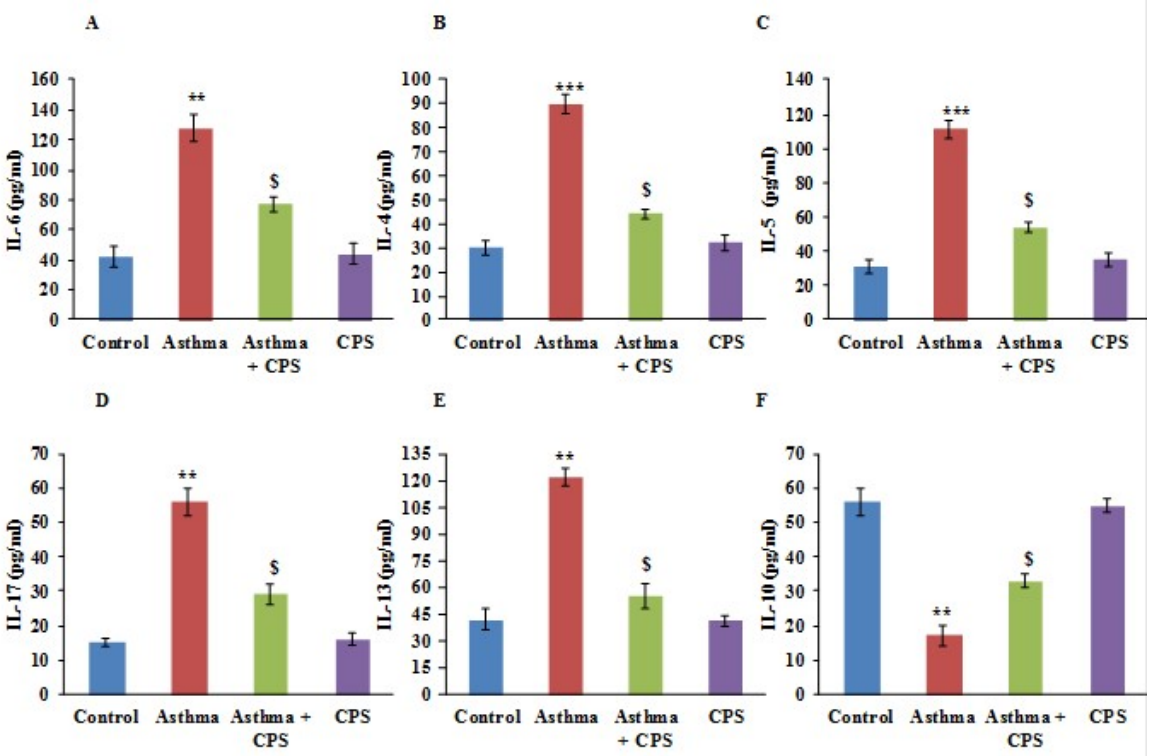

Fig. 3: A-F represents cytokine expression analysis of IL-4, IL-5, IL-6, IL-13, IL-17, and IL-10 in the control and experimental group of rats. The experimental details were given in the methodology section. Values are expressed as mean ± S.E (n = 8). Statistical significance expressed as **p<0.01, ***p<0.001 compared to vehicle-treated controls, $p<0.05 CPS compared to asthma rats; ns denotes non-significant.

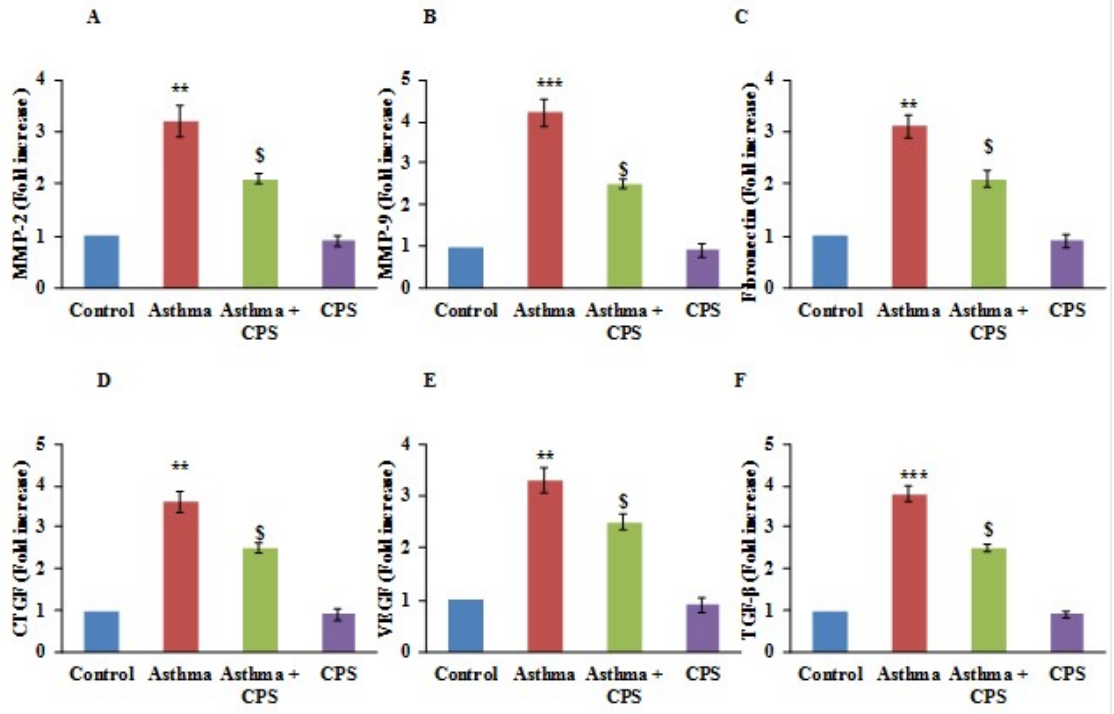

Fig. 4: A-F represents qRT-PCR mRNA expression analysis of MMP-2, MMP-9, Fibronectin, VEGF, CTGF, TGF-β in control and experimental group of rats. The qRT-PCR experimental details were given in the methodology section. Values are expressed as mean ± S.E (n = 8). Statistical significance expressed as **p<0.01, ***p<0.001 compared to vehicle-treated controls, $p<0.05 CPS compared to asthma rats; ns denotes non-significant.

asthmatic animal group was treated with CPS and the effect of nitrotyrosine gets significantly reduced with the reduction in inflammatory and lung cells' reactivity to nitrotyrosine.

The pulmonary function tests performed in the asthmatic models, especially, showed that the animals are under broncho-constriction. They are measured for understanding the mechanical properties of the airway and tissues in asthmatic as well as CPS-treated animals. Respiratory resistance is significantly high in the case of asthmatic animals compared to the controls. The values decrease with respect to the response given for CPS treatment in the other group. These effects are due to the changes that are observed with regard to the smooth muscle activity (Que *et al*., 2001) and stiffness as a result of the structural changes that happen due to airway remodeling and hypertrophy (Mendonca *et al*., 2011). The conditions would worse with contraction in the airway due to hyperresponsiveness in the extreme cases of asthma.

The airway inflammation is often accompanied by an upsurge in the expression of pro-inflammatory cytokine IL-6 and increased expression of Th2 cytokines such as IL-4, IL-5 (Doherty and Broide, 2007), IL-13 in the lung (Jeffery, 1999). An increase in the IL-4 and IL-13 means asthma-mediated inflammation in the airway has set and it has undergone remodeling of the airway and acute hyperresponsiveness due to mucus accumulation has occurred. These increases are specific to the Th2 - mediated response observed in the asthmatic animals. A simultaneous increase in the goblet cells which are responsible for mucus secretion (Thomas, 2001) (Doran *et al*., 2017) has occurred, and IL-13 is responsible for the survival of eosinophils, activation of them and then trafficking them to the site of inflammation. Hence, IL-13 plays a chief role in the pathogenesis of asthma in the OVA-induced animals with IL-6 promoting the production of IL-13 (Neveu *et al*., 2009). In the present study, treatment with CPS has increased the anti-inflammatory molecule IL-10 in effectively controlling the inflammation.

In order to find the molecules behind the pathogenesis of airway remodeling in asthma, we have elucidated the levels of growth factors that are expressed in our model. An increase in the airway vasculature is observed in cases of asthma (Li and Wilson, 1997, Tanaka *et al*., 2003) with an increase in the vascular endothelial growth factor (VEGF) (Hoshino *et al*., 2001) which would encourage new vasculature. Due to this, a surge in the inflammatory cytokines and molecules involved in airway remodeling would occur into the airway walls and results in structural alterations of the airway walls. Next, we have observed an increase in the expression of Connective tissue growth factor (CTGF) which is known to induce cell migration, cell adhesion, cell proliferation and synthesis of ECM (Moussad and Brigstock, 2000) in asthmatic animals. It is expressed highly in the airway smooth muscle cells of asthmatic animals and induces the cells to proliferate faster and release fibronectin and collagen I (Black *et al*., 2003, Burgess *et al*., 2003, Johnson *et al*., 2006).

The deposition of extracellular matrix protein, TGF-beta1, fibronectin underneath the epithelium (Huang *et al*., 1999, Roche *et al*., 1989) would result in the thickening of the basement membrane and is a significant alteration in the airway remodeling. This subepithelial fibrosis is a feature that is observed in all asthma extremities (Boulet *et al*., 1997, Elias *et al*., 1999) and an increase in the airway thickness is featured (Little *et al*., 2002). Under these situations, the balance between ECM proteins production-degradation (Bergeron *et al*., 2009) is not maintained, and the levels of matrix metalloproteases tilt in favor of fibrosis (Bergeron *et al*., 2009). MMP-9 is the major contributor of fibrosis mediation in asthma, and their levels were found to be increased in the asthmatic rats (Suzuki *et al*., 2001, Vignola *et al*., 1998). It affects the matrix and reorganizes and causes airway inflammation through eosinophil infiltration, revascularization (Johnson *et al*., 2004) and smooth muscle hyperplasia (Johnson and Galis, 2004). In our study, we have detected that MMP-2 and MMP-9 got increased in the asthmatic rats and these proteases play a major role in the degradation of ECM in vascular remodeling (Lu *et al*., 2011) (Vitlianova *et al*., 2015). The induction of these smooth muscle cells of the airway by these MMPs is a significant step in the active remodeling of the airway (Nishihara-Fujihara *et al*., 2010). The coordination of CTGF in increasing the expression of MMP-9 has been found much evidence (Chintala *et al*., 2012, Fan and Karnovsky, 2002) and they together found a greater role in the asthmatic airway remodeling.

The overall composition of the airway constitutes an important part of the airway remodeling, and that is contributed by various factors in combination to give a structural rearrangement from the accumulation of ECM to neovascularization. Other report has observed that eosinophilic infiltration has increased in the asthmatic subjects and that eosinophils increase the expression of VEGF (Hoshino *et al*., 2001) is an important observation that correlates our findings that eosinophils also increase in our asthmatic animals and that along with smooth muscle cells contribute to the angiogenesis through VEGF in the airway remodeling.

CONCLUSION

The present findings clearly say that airway smooth muscles are at the forefront of airway remodeling with additional contributions from the eosinophils and growth factors arising out of airway inflammation. These effects were high in the asthmatic animals, and it can be

controlled with the use of CPS. These effects of our molecule would be sustainable and would be a potential candidate for its use in controlling the allergen-induced asthmatic effects of inflammation and its subsequent airway remodeling which is difficult to be cured with beta-agonists and corticosteroids. With the present results obtained in testing animal models and further research for its use in human subjects especially in neo-natal would be giving hope for the fight against asthma.

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