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Inhibition of Airway Contraction and Inflammation by Pomalidomide in a Male Wistar Rat Model of Ovalbumin-induced Asthma

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ABSTRACT

Asthma is a chronic inflammatory disease of the airways of the lungs. Pomalidomide (POM) a therapy for multiple myeloma has been stated to have an anti-inflammatory effect. The main goal of the present study was to assess its possible effect on airway contraction and inflammation in a rat model of ovalbumin-induced asthma.

Different groups of rats received saline or pomalidomide (0.4, 0.8 mg/kg) or dexamethasone (0.6 mg/kg). The asthma was induced by ovalbumin (OVA). Trachea contraction was assayed by organ bath system. Airway histology was assessed using hematoxylin and eosin method. Serum Tumor necrosis factor alpha (TNF-α) level was analyzed by Enzyme-Linked Immunosorbent Assay and Platelet-derived growth factor (PDGFα) Gene expressions were evaluated by Real-time PCR.

Pomalidomide prevented ovalbumin-induced airway contraction and histopathological damage. In addition serum, TNF-α level was significantly (p<0.05) decreased in POM treated animals compared to control (asthmatic animals that received POM vehicle). Results indicate that POM prevented the PDGF expression induced by ovalbumin.

In conclusion, we found that pomalidomide ameliorated the symptoms, histopathological changes and inflammatory markers induced by ovalbumin in asthmatic rats and these effects might be related to its anti-inflammatory properties.

Keywords: Asthma; Inflammation; Platelet-derived growth factor; Pomalidomide; TNF- α

INTRODUCTION

Asthma is specified by construction changes at airway that possibly will lead the extension of asthma, containing sub-epithelial fibrosis, epithelial damage, and increased smooth muscle mass.[1,2] The principal mechanism of asthma is airway inflammation and altering the structure of the airway with chronic effects

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to pulmonary function.[3] In addition, according to the previous studies there is a positive relationship between the thickness of the lung airway smooth muscle (ASM) layer with airway responsiveness.[4] Its common features including augmentation of mast cells, lymphocytes and eosinophils, and mediators such as interleukin-3 (IL-3), IL-4, IL-5, and IL-13.[5] Eosinophil is an important factor and the number of eosinophils is higher in allergic pulmonary disease.[6,7] Some asthmatic patients have diverse forms of inflammation, consisting neutrophilic bronchitis or, less frequently, other inflammatory cells.[8] Antioxidant agents might reduce eosinophil infiltration and mucus overproduction in ovalbumin(OVA)-exposed lung tissues[9] and B cells are a major factor of the inflammation associated with long term allergic disease.[10]

Tumor necrosis factor alpha (TNF-α) is an important pro-inflammatory cytokine and activator of inflammatory cells.[11] Some evidence have shown that TNF-αplays a key role in asthma severity.[12]

Previously, attention has been focused on the increased expression of platelet-derived growth factor (PDGF)in lung inflammation.[13,14] PDGF is a growth factor released by macrophages in fibrosis,[15,16] and its mRNA was reported to be existing in macrophages from normal cases and asthmatics.[17] In addition, it has been reported that PDGF is involved in lung fibrosis[13-15] and fibroblasts are stimulated in asthma.[18] In asthma, there is a rise in smooth muscle mass[19-22] and PDGF is one of the smooth muscle growth factors.[23] PDGF presents in tissue repair of chronic inflammatory diseases of the airways with repair injury via proliferation and migration of connective tissue cells.[23,25]

Pomalidomide (POM) is an anti-inflammatory agent with the ability to inhibit the synthesis of TNF-α. It is the new structure of thalidomide that has discovered in 1996. According to the data, its anti-inflammatory and TNF-α inhibitory effect is about a 20,000 times stronger than thalidomide.[26,27] It is also shown that pomalidomide prevent other pro-inflammatory cytokines, such as IL-6, IL-1b, and IL-12, and augment the creation of the anti-inflammatory IL-10, by lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs).[28,29]

Though several clinical experiment has been performed by pomalidomide to cure advanced myelodysplastic syndrome, multiple myeloma and metastatic prostate cancer, but any of these studies not by using pomalidomide to treat inflammation-associated diseases. Moreover, another study, on the effects of pomalidomide in pancreatitis showed that pomalidomide reduced symptoms of the disease by reducing TNF-a, IL-1b.[30]

According to the above information, the main purpose of the present evaluation was to investigate the effects of pomalidomide on airway contraction and inflammation in a rat model of ovalbumin-induced Asthma.

MATERIALS AND METHODS

Animals

Six-week-old male Wistar rats weighing 250±20 were bought and kept in the standard conditions of temperature, brightness, and darkness. Animals had access to food and water ad libitum. All experimentations were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Science and Research Branch, Islamic Azad University Animal Care and Use Committee (No.2018- 97362).

Ovalbumin, dexamethasone, and pomalidomide were purchased from Sigma Aldrich, Germany. The above doses of ovalbumin, pomalidomide and dexamethasone were selected based on previous observations.[31] To determine the maximum therapeutic effect, two doses of pomalidomide (0.4, 0.8 mg/kg) were used.[32,33]

Table 1. Male Wistar rats divided into 5 groups in a study on airway contraction and inflammation assessment (n=8 rats per group)

Group	Treatment details
Saline	Normal Saline (1 mL/kg, po)
OVA+Saline (control)	Ovalbumin (5 µg/kg , ip)+Normal Saline (1 mL/kg, po)
OVA+Dex	Ovalbumin (5 µg/kg, ip)+Dexamethason (0.6 mg/kg, ip)
OVA+Pom 0.4	Ovalbumin (5 µg/kg, ip)+Pomalidomide (0.4 mg/kg, ip)
OVA+Pom 0.8	Ovalbumin (5 µg/kg, ip)+Pomalidomide (0.8 mg/kg, ip)

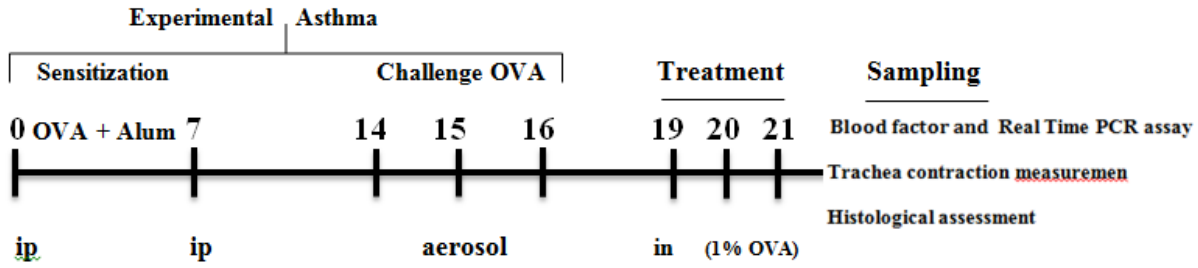

Figure 1. Schematic diagram of the groups design and protocol for the rat model of ovalbumin-induced asthma

The asthma model induced in animals according to the previous studies is illustrated in Figure 1.[34]

Histological Assessment

The airway was isolated from the animals 24hours after the last OVA administration. Samples were fixed in 10% formalin, embedded in paraffin, and cut into 4-µm pieces. The samples were positioned on glass slides, deparaffinized, and stained with hematoxylin and eosin (H&E). In addition, some samples were treated for Masson's trichrome stain (HT15; Sigma, USA) to visualize collagen fibers and smooth muscles. Airway inflammation was described as the sum of the peribronchial and perivascular scores.[35,36]

Rat trachea Contraction Measurement

Rats were anesthetized using ketamine/xylazine cocktail. Under surgery, about one centimeter of animal trachea was isolated. In the start of an experiment, the tracheal rings were suspended in organ bath chambers (Panlab Harvard Apparatus, Holliston, Massachusetts, USA) covering oxygenated (95% O2; 5% CO2) Krebs solution maintained at 37°C. Kreb's solution consisting of (mmol/l) NaCl, 118; KCl, 4.7; CaCl2, 1.2; KH2PO4, 2.5; MgSO4·7H2O, 1.2; NaHCO3, 25.0; and glucose, 10.0.Variations in isometric tension were analyzed by attaching the upper end of the ring to an isometric tension transducer (MLT0420, AD Instruments, and Australia) and recorded on AD Instruments Power Lab Chart software. Contractions trachea registered by physiographic with speed 0/1 (mm/s).

Serum TNF-α Content Assay

Animals were anesthetized (ketamine-xylazine cocktail) and five-milliliter blood samples were collected from the heart after animals were sacrificed. Subsequently, serum TNF-α level was assayed using specific rat ELISA kits, according to the manufacturer's instructions. These findings were expressed as absolute (ng/mg of protein). Protein content was assessed using Lowry's method with bovine serum albumin (BSA) as a standard.[37]

Extraction of Total RNA, cDNA Synthesis and Real Time PCR for PDGFα

Total RNA was extracted from the serum (5 mL blood from male Wistar rats in a tube containing cold EDTA) that was kept -20°C for 24 hours. For processing, they were first returned to ambient temperature, and then centrifuged at 3000×g for 10 minutes at a rotary surface, and twice to remove the supranates, 4 ml of sterile PBS was added to the test tube and then significantly suspended. A suspension of 3000×g centrifuges was used. Then, with 400 µL of TRI and 30 ml of Proteinase K, it was transferred to a microcentrifuge tube and rotated for 5 seconds at 55°C for 10 minutes in a shaker (500 rpm). Then, about 43 µL of chloroform was added and the samples vortexed for a short period of time. After incubation for 5 minutes at ambient temperature, the sample tube was centrifuged for 12 minutes at 4°C. The upper layer of water (100 to 200 µL) was transferred to a microcentrifuge tube and stored at -70 ° C. Then, using MagMAX automated RNA extraction, RNA was extracted and tested. After determining the concenter RNA. The cDNA was synthesized using Template RNA, primer F, primer R, mastermix and H2O; inserted into the PCR to maximize synthesis and was synthesized during different cycles of CDNA. To detections mRNA expression, the following F primers from sense, 5' TGTAACACCAGCAGCGTCA3'and R primers antisense, 5'GGGTTCAGG TTGGAGGTGC 3'. For PDGFα were used. To analyze the results for Bio-Rad (RT-PCR) protocol using of expressions PDGFα mRNA, Was used ΔΔCT. Reactions were performed in a 10 µL volume with 0.125 µM primers

Sample groups separately, 2.5 mMMgCl2, nucleotides, buffers, Taq DNA polymerase and DNA Master SYBR Green I mix were used. Melting curve analysis indicates the quality of the test steps. The Real-Time PCR cycles were at 62.5 Celsius for 2:05 hours done, and include the baseline region, the exponential phase, linear phase and plateau phase recorded in 45 cycles. GAPDH used for Housekeeping real time in this protocol.

Statistical Analysis

Data were analyzed by the IBM SPSS Statistics, version 19 (IBM Corp., Armonk, N.Y., USA). All quantitative parameters were presented as mean±SEM. The difference between groups was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test for percentage of tissue contractions, the index below the histogram data obtained from the tissue bath and also the finding of ELIZA and changes in the gene expression. The p <0.05 were considered to be significant in all analyses.

RESULTS

Pomalidomide Significantly Reduced Airway Contraction

Statistical analysis revealed that the area under the curve of the contraction graph was dramatically increased in asthmatic rats and was significantly more than the healthy control group (p=0.045). Real chart from contraction measurement of rat trachea showed in Figure 2. Also, daily dexamethasone administration significantly prevented OVA-induced change in the area under the curve. In addition, airway contraction was almost the same as the healthy group in pomalidomide (0.1, 0.4 mg/kg, daily) received animals (Figure 3).

Effect of Pomalidomide on Serum TNF-α Level in Ovalbumin-Induced Asthma

The results in Figure 4 indicate that serum TNF-α levels were significantly higher in OVA-challenged rats in comparison with healthy control. On the other hand, pomalidomide prevented ovalbumin-induced TNF-α augmentation. In fact, this index was significantly lower in Pom-received than asthmatic rats and it was similar to the control group. In addition, the results showed that pomalidomide was as effective as dexamethasone as a positive control group (Figure 4).

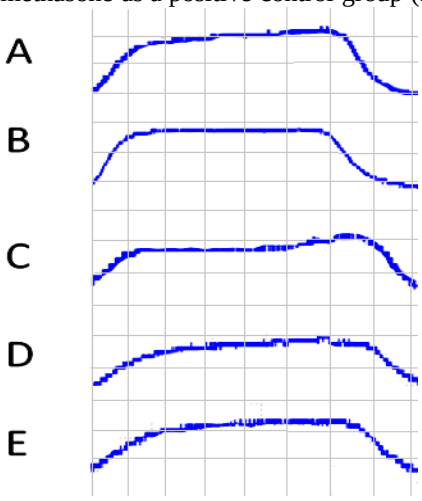

Figure 2. Real chart contraction measurement of rat trachea in a study on airway contraction and inflammation assessment A: Normal Saline, B: Normal Saline+Ovalbumin, C: Ovalbumin+Dexamethason, D: Ovalbumin+Pomalidomide (0.4 mg/kg), E:Ovalbumin + Pomalidomide (0.8 mg/kg)

Table 2. Male Wistar rats airway contraction and PDGFα gene expression in the study groups

Group	Saline	OVA+Saline (control)	OVA+Dexa	OVA+Pom 0.4	OVA+Pom 0.8
contraction (%)					
the area under the curve	0.53± 0.02	0.84±0.01*	0.32±0.02*#	0.61±0.03*#	0.43±0.03*#
(10 2 index)	55.95± 4.81	79.73± 3.55*	49.48±1.13 *#	54.96±2.49#	52.16±3.3#
ΔΔCT					
(fold change gene expression)	0.53± 0.02	0.84±0.01 *	0.32±0.02*#	0.61±0.03*#	0.43±0.03*#

All data points are expressed as mean±SEM in five groups including Saline, OVA+Saline (control), OVA+Dexa, OVA+Pom 0.4 mg/kg, OVA+Pom 0.8.*p<0.05 significantly different versus healthy animals (saline group). # p<0.05 significantly different versus control (OVA+Saline) group

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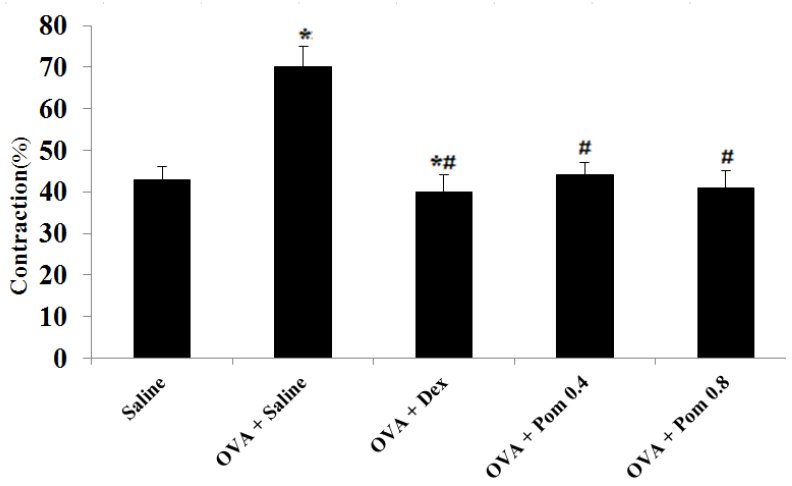

Figure 3. Effects of pomalidomide on rat trachea contraction in the study groups including Normal Saline, Ovalbumin / Normal Saline, Ovalbumin/Dexamethason, Ovalbumin/Pomalidomide (0.4 mg/kg) and Ovalbumin/ Pomalidomide (0.8 mg/kg) using set isolated tissue. All data points are expressed as contraction mean (%)±SEM in five groups.*p<0.05 significantly different versus healthy animals (saline group). # p<0.05 significantly different versus control (OVA+Saline) group.

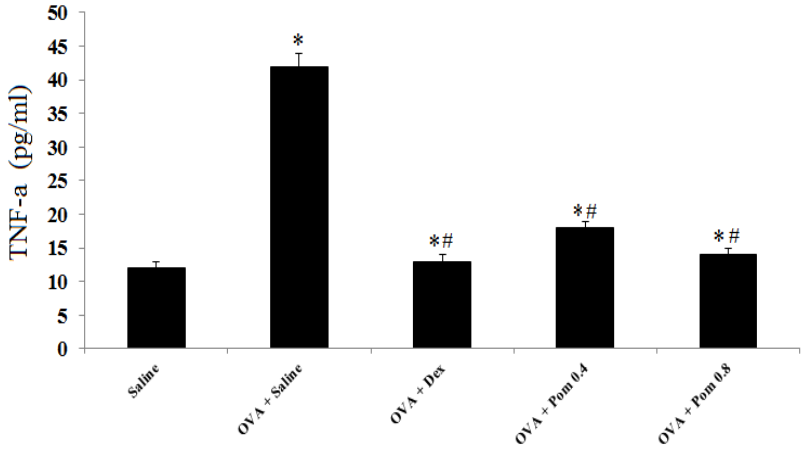

Figure 4. Effects of pomalidomide on TNF-α level and the Airways by allergen challenges in the study groups including Normal Saline, Ovalbumin / Normal Saline, Ovalbumin / Dexamethason, Ovalbumin/ Pomalidomide (0.4 mg/kg) and Ovalbumin/ Pomalidomide (0.8 mg/kg) . All data points are expressed as mean±SEM in five groups..*p<0.05 significantly different versus healthy animals (saline group). # p<0.05 significantly different versus control (OVA+Saline) group.

Effect of Pomalidomide on OVA-Induced Airway Histological Changes

The effects of pomalidomide on OVA-induced airway histological were determined. Total respiratory resistance was increased in OVA-challenged rat as compared with control rat. In Group (a, b) (Figure 5), the histological assessment showed better compared to the group OVA-challenged rat (c, d). When low and high dosages of pomalidomide were compared to each other; all histological findings showed to be better in the group treated with higher dose of pomalidomide (Figure 5) but for a dose-response conclusion, more doses are needed to be examined. Also, some samples were treated for Masson's trichrome stain (HT15; Sigma, USA) to visualize smooth muscles (Figure 6).

Pomalidomide Reduces PDGFα Gene Expression

The findings this study showed that pomalidomide and dexamethasone reduced PDGFα gene expression in the asthmatic rat. Ovalbumin significantly induced compared to the control group (p=0.04). While administration of pomalidomide and dexamethasone prevented PDGFα gene fold changes induced by ovalbumin and keep it even less than control (Figure 7).

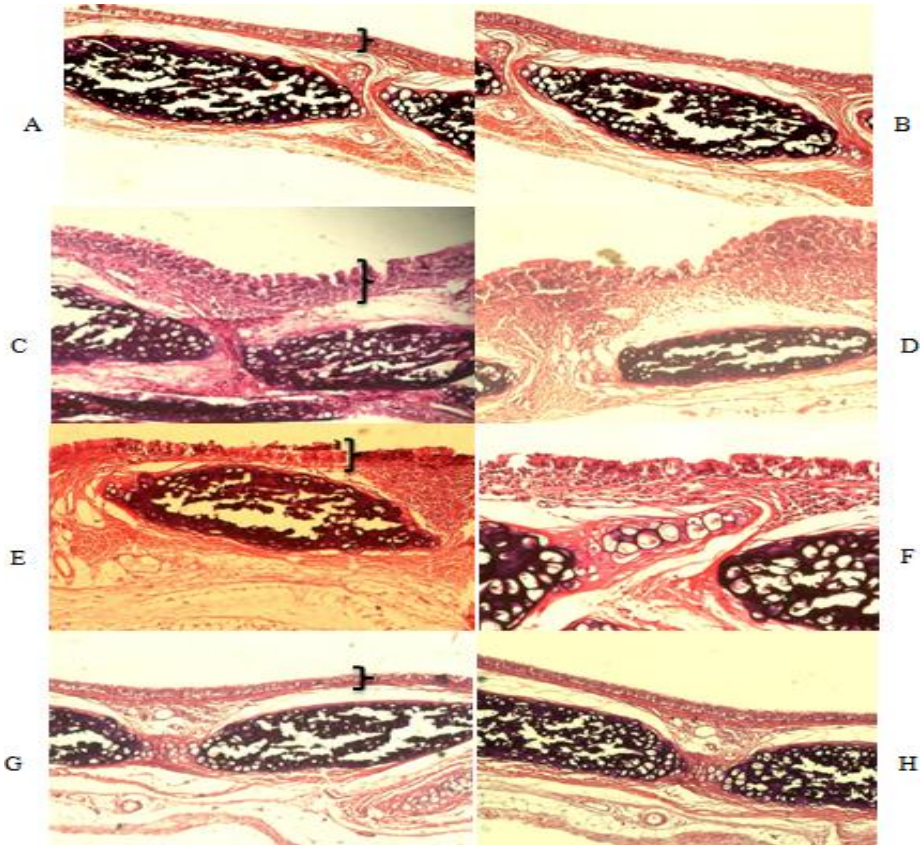

Figure 5. Effects of Pomalidomide on inflammatory airway smooth muscle. Histological analyses of lung tissue were achieved for the Inflammatory airway smooth muscle in saline (A, B), Normal Saline+Ovalbumin (control) (C, D), Ovalbumin+Pomalidomide (0.4 mg/kg) (E, F) and Ovalbumin + Pomalidomide (0.8 mg/kg) (G, H). (up ×100 , down:×40).

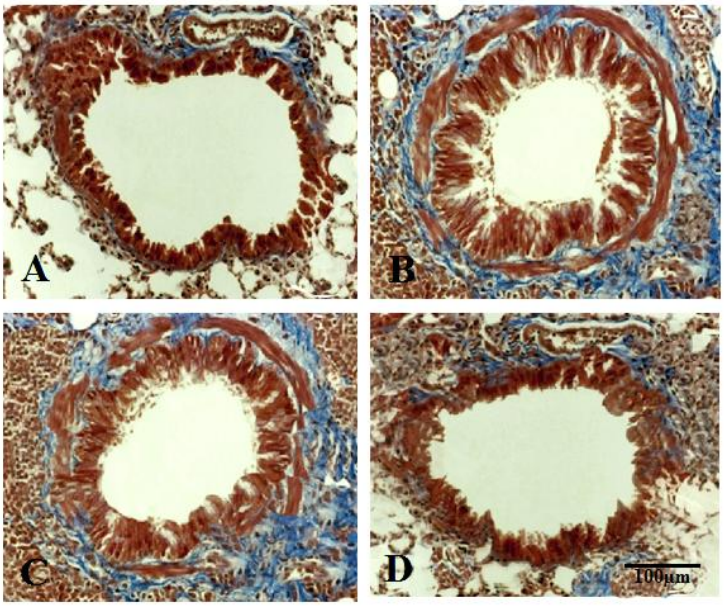

Figure 6. Effects of Pomalidomide on inflammatory airway smooth muscle mass. Lung tissue was obtained on Day 21 from Saline (A), Normal Saline+Ovalbumin (B), Ovalbumin+Pomalidomide (0.4 mg/kg) (C), Ovalbumin+Pomalidomide (0.8 mg/kg) (D), and stained with Masson's trichrome stain.

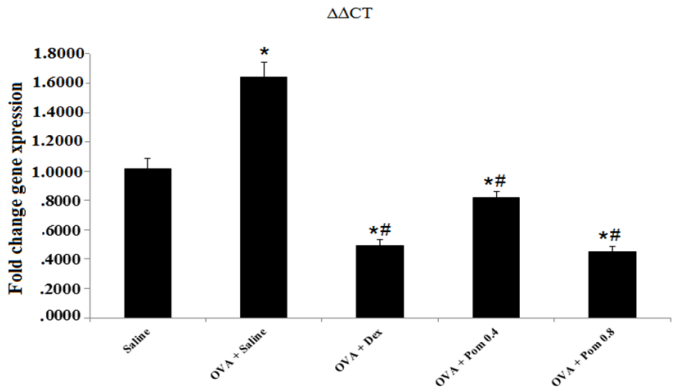

Figure 7. Effects of pomalidomide on PDGFα gene expression level and the airways by allergen challenges in study groups including Normal Saline, Ovalbumin / Normal Saline, Ovalbumin / Dexamethason, Ovalbumin/ Pomalidomide (0.4 mg/kg) and Ovalbumin/ Pomalidomide (0.8 mg/kg). All data points are expressed as mean±SEM in five groups.*p<0.05 significantly different versus healthy animals (saline group). # p<0.05 significantly different versus control (OVA+Saline) group.

DISCUSSION

This is the first report regarding the protective properties of pomalidomide against lung inflammation in the OVA-induced rat model of asthma. We found that Pom-treated animals showed significantly fewer inflammatory indices in airway and lung tissues and reduce the expression of PDGF.

Asthma is specified by construction changes at airway and fibrosis, epithelial damage, increased smooth muscle mass, lung inflammation and subsequently obstruction of the airways.[1] Based on our findings in the present study, contraction measurement of rat trachea showed that smooth muscle mass and airway hyper-responsiveness were significantly decreased by pomalidomide treatment. Airway hyper-responsiveness was significantly decreased in pomalidomide (0.8 mg/mL) treatment as compared with pomalidomide (0.4 mg/mL).In addition, according to the previous studies, there is a positive relationship between the thickness of the ASM layer with airway responsiveness also between the regulator of inflammatory cells and the level of airway hyper-responsiveness.[4,38]

In addition, histological H&E staining and Masson's trichrome analysis of the airways tissues displayed that there was additional deposition of collagen in the asthmatic rat administered with the pomalidomide treatment. Several lines of evidence demonstrated too much production of mucus and collagen and thus augmentation the thickness of airway wall as one of the important symptoms that occur in asthma. The results of the present investigation were in agreement with the results of Asano et al study who revealed that the hyperplasia of goblet cells was considerably inhibited by thalidomide, an agent with less anti-inflammatory effects than pomalidomide.[39]

TNF-α is a pro-inflammatory cytokine and does as an activator and regulator of inflammatory cells.[11] And plays a key role in asthma severity.[8] Therefore, TNF-α inhibitors such as thalidomide reduced symptoms of asthma through suppression of this cytokine.[39] Previously it has been reported that pomalidomide is about a 20,000 times stronger than thalidomide as a TNF-α inhibitor.[27,40] Accordingly, the results of this study showed that pomalidomide significantly decreased TNF-α level. Confirming these results, Lee and colleagues in 2015, showed that thalidomide significantly decreased TNF-α level.[41] In another study, it was reported that pomalidomide ameliorated the symptoms of cerulean-induced acute pancreatitis through reducing TNF-α and IL-1b.[30]

In addition here we found that pomalidomide could prevent OVA-induced elevation of PDGF, which is a growth factor released by macrophages in fibrosis,[12] and these cells being increased in asthma.[42,43] PDGF is one of the smooth muscle growth factors[23] and it is well known that there is an escalation in smooth-muscle mass in asthma.[19-22] In this regard, Liang and colleagues showed airway remodeling and inflammation by isoforskolin inhibited in PDGF

induced rat ASMCs and OVA-induced rat asthma model.[44] Also, PDGF is involved in tissue reparation of prolonged inflammatory diseases of the airways including repair injury via proliferation and migration of connective tissue cells.[23,25] According to the results pomalidomide was as effective as dexamethasone as a potent anti-inflammatory agent in this effect.

In this study, we found that pomalidomide 0.8 mg/kg was more effective than 0.4 mg/kg in cellular and molecular assessments. However, additional investigations are required to validate effective dose of this drug in asthma.

There were some limitations in this study including not dose-dependent evaluation, chronic administration of pomalidomide and possible side effects. In fact in this kind of basic studies researchers are trying to find new agents or at least a new application for current drugs at sub-therapeutic doses to apply them as anti-inflammatory agents for respiratory disease. Surely for clinical application more studies using different doses, time-points and evaluations of side effects of drugs are needed.

In conclusion, we found that pomalidomide inhibited the airway contraction and inflammation in an animal model of ovalbumin-induced asthma and it seems that these effects mediated through interfering serum TNF-α level and PDGF gene expression.

ACKNOWLEDGEMENTS

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