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Myrcene exerts anti-asthmatic activity in neonatal rats via modulating the matrix remodeling

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Abstract

Myrcene (MC), an organic hydrocarbon, was found to exert anti-inflammatory, analgesic, antimutagenic and antioxidant properties. However, the protective role of MC has not been reported against neonatal asthma. Wistar rats induced with asthma were administered with MC; while asthma control and vehicle control were maintained without MC administration. At the end of the experimental period, lung histology, inflammatory cell counts, cytokine analysis, matrix protein expressions were elucidated. Rats administered with MC exerted significant ($P < 0.05$) defense in protecting the lung tissue with the evidenced restoration of alveolar thickening of the lung tissues. Also, the present study elicited the anti-asthmatic activity of MC, especially via modulating the extracellular matrix protein expression in the asthma-induced animals, while a significant reduction ($P < 0.05$) in the fibrotic markers were found in MC treated animals. Moreover, the protective effect of MC was evidenced with reduced leukocyte infiltration in BALF, hypersensitive specific IgE levels with a profound decrease in the inflammatory cytokines such as IL-2, IL-4, IL-18, and IL-21 in MC administered animals compared to the asthma-induced group. To an extent, the markers of asthmatic inflammation such as CD14, MCP-1, and TARC were also found to be attenuated in MC exposed animals. The possible application of MC is a promising drug for the treatment of asthma-mediated complications.

Keywords

anti-asthmatic activity, matrix remodeling, myrcene, neonatal rats

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Introduction

Asthma is a common inflammatory disease that affects the lungs and people worldwide are affected in millions due to this obstructive lung disease.[1–3] Important advancements have been made in the drugs used for therapy on the care, and its prevalence has not decreased much in recent decades. Although the mortality rates are considerably low, it can be avoided in many cases due to routine care.[4] But it has been projected to be the leading cause of death worldwide due to the incidence of industrialization and rapid change in the weather conditions of the earth that the pollutants and asthma causing agents can travel to far off places. It would cause impairment in the quality of life physiologically.

The major symptoms of asthma include bronchial hyper-responsiveness, increased mucus production and narrowing of airways and its remodeling which are due to the infiltration of the immune cells into the lungs and the subsequent consequences causing lung inflammation. Due to

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this, the patients may exhibit narrowing of the airways and accumulation of the mucus causing shortness of breath, chest discomfort, wheezing, and cough.[5,6]

Anti-inflammatory medications that are taken orally and inhaled are the usual bronchodilator medications that are recommended for asthma[7] and the associated chronic obstructive pulmonary disease (COPD), has been used for decades and are not without any side effects.[8] Hence there is a need to consider the alternative medicines that are based on phytochemicals.

Myrcene is an acyclic monoterpene found in the essential oil of lemongrass, hop, mango, verbena, bay, and cannabis. It is volatile and is widely used in the fragrance industry.[9] It is a non-mutagenic compound[10] and is generally regarded to be safe and is used in traditional medicines as a pain killer. The anti-nociceptive properties of myrcene have been already demonstrated.[11,12] The production of anti-inflammatory cytokines is mediated by myrcene and is active against inflammation[13] due to external agent induction. It is known to exert its immunoregulation activity by inhibiting the production of NO and IL-4 that are produced during lung inflammation.[14,15] The anti-inflammatory property of myrcene has been used in our experimental model in which ovalbumin (OVA) was used to induce asthma and after treatment with myrcene (MC), we have evaluated the effect of MC in controlling the asthma by decreasing the expression of pro-inflammatory mediators and reducing the effect of lung fibrosis.

Materials and methods

Chemicals

Ovalbumin (chicken), ß-myrcene (~90% purity) were obtained from Sigma Aldrich, USA. RNA isolation kits, cDNA synthesis, and SYBR Green/ROX master mix were from Thermo Fischer Scientific, USA. Primer sequences for PCR were obtained from Eurofins MWG (Operon). Assay kits for IgE, histamine, and nitrotyrosine were from Abcam Inc, USA. ELISA assay for IL-2, IL-3, IL-4, IL-18, IL-6, and IL-21 were from Fine test biotech, China. MMP-2, MMP-9, TGF-β1, Collagen-1 assay kits are from Cusabio, USA. Periostin, CXCR2 ELISA kits were from Abcam Inc, USA. All other chemical reagents used were analytical grade.

Asthma experimental animal model

Wistar rats (8–10 g) were used as a model in the study. The animals were maintained acrylic plastic cages in air-conditioned rooms (22°C, humidity 60%, 12 h each day, and night light cycles were maintained). Pellet food and tap water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee, and the procedures were followed strictly as per the guidelines of the committee. Wistar rat pups of 10 days old were used in the current investigation and grouped as follows. Vehicle-treated as control group, Asthmatic group (OVA administered) as an induced group, asthma rats treated with myrcene (MC) (25 mg/kg dissolved in peanut oil (pharma grade), oral administration, and daily, before the OVA exposure) as the treatment group, and MC drug-control group. The vehicle-control group rats received a similar treatment without MC (peanut oil, 2.5 mL/kg body weight alone).

Experimental analysis

For the experimentation, the pups were sensitized with intraperitoneal administration of OVA (20 µg) on day 1 and 7th and from the 14th until 28th days along with 30 min aerosol exposure of 1% OVA from 14th day onwards for 2 weeks.[16] At the end of the experimental period, the animals were killed, bronchoalveolar lavage fluid **(**BALF) was collected to quantify infiltrating inflammatory cells[17] and blood was collected by cardiac puncture. Also, the lung tissues were collected for hematoxylin and eosin staining and Masson's trichrome staining for collagen accumulation and the fibrosis was scored compared to the control group.[18] Further, asthma specific IgE levels, histamine, and nitrotyrosine were estimated using commercial assay kits as per the manufacturer's instructions.

Estimation of inflammatory cytokines

The assessment of pro-inflammatory and anti-inflammatory cytokines such as IL-2, IL-3, IL-4, IL-18, IL-6, and IL-21 in the serum samples and BALF samples was estimated using commercial ELISA kits as per the manufacturer's instruction.

Table 1. List of primers used in the study.

Gene	Primer	Sequence (5′-3′)	Annealing	Accession number
Fibronectin	F	TCCACCTGTACACGCTCAAC	59	XM_032901311.1
	R	GGGTGTGGAAGGGTAACCAG		
CTGF	F	GGGAGTCAGGTGACACGAAC	59	AB023068.1
	R	CACACACCCAGCTCTTGCTA		
CD-14	F	GTTGGGCGAGAAAGGACTGA	58	XM_032886019.1
	R	GTTATACGCCTCCGACTGGG		
MCP-1	F	GATCCCAATGAGTCGGCTGG	57	M57441.1
	R	ACAGAAGTGCTTGAGGTGGT		
TARC	F	TGATGTCACTTCAGATGCTGCT	58	NM_057151.1
	R	TCTGTGCAGATAAGCCTTCCC		
IFN-γ	F	CAGGCCATCAGCAACAACAT	59	XM_032890807.1
	R	GGCACACTCTCTACCCCAGA		
GAPDH	F	GCATCTTCTTGTGCAGTGCC	59	XM_032902285.1
	R	GATGGTGATGGGTTTCCCGT		

Further, the fibrosis markers such as MMP-2, MMP-9, TGF-β1, collagen-1, periostin, CXCR2 were also estimated using commercial ELISA kits.

Reverse transcription-PCR

For the elucidation of asthma-related genes, the total RNA was isolated from the lung tissues using TRIzol reagent. Briefly, the tissues were homogenized using TRIzol, and the homogenate was mixed with chloroform and centrifuged for 10,000 rpm for 20 min, 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 using a spectrophotometer. The purity of RNA was analysed using the 2% formaldehyde agarose gel electrophoresis. About 20 μL total RNA (quantified and equal amount from all groups) 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 study 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 a control-house-keeping gene.

Statistical analysis

Statistical significance was assessed using Graph pad prism software. The statistical analysis was performed using a student *t*-test, and the differences between groups with a *P*-value of less than 0.05 were considered statistically significant.

Results

The present study aimed at elucidating the protective role of MC against neonatal. From the in vivo experiments, the results of lung histology demonstrated that asthma induced rats displayed the characteristic hypertrophy with the accumulation of smooth muscle mass in the mucous gland compared to control. Meanwhile, the analysis of collagen accumulation demonstrated a 3-fold increase in collagen accumulation in asthma induced animals. On the other hand, MC treatment displayed a significant reduction in smooth muscle matrix accumulation with reduced collagen accumulation (Figure 1) demonstrating the protective effect of MC against asthma.

Figure 2 represents the analysis of the infiltration of eosinophils into BALF, and the results demonstrated a significant number of cells were infiltrated into the BALF compared to control. While, the hypersensitivity based IgE levels, histamine, and nitrotyrosine were profoundly increased in asthma-induced rats. However, animals pretreated with MC displayed a significant reduction in the asthmatic metabolites (Figure 2).

Additionally, the analysis of the cytokines' levels in control and experimental of rats is presented in Figure 3. The results displayed that the asthma rats established a significant increase in the levels of cytokines such as IL-2 ($P < 0.001$), IL-3 ($P < 0.001$), IL-4 ($P < 0.001$), IL-18 ($P < 0.001$), IL-6 ($P < 0.001$), and IL-21 ($P < 0.001$) compared

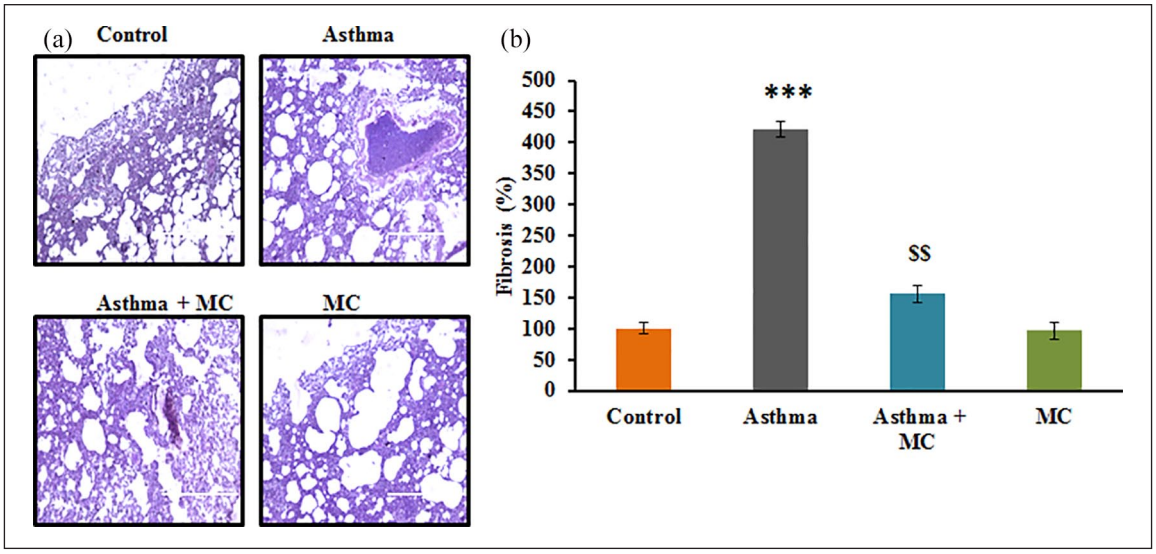

Figure 1. (a) Lung tissue histology stained using hematoxylin and eosin stain. (b) The fibrosis score of the control and experimental group of rats. The details of staining and scoring were given in the methodology section. Values are expressed as mean ± standard error (SE) (n = 10). Statistical significance expressed as ***$P < 0.001$ compared to vehicle-treated controls; $$ $P < 0.01$ compared to asthma rats.

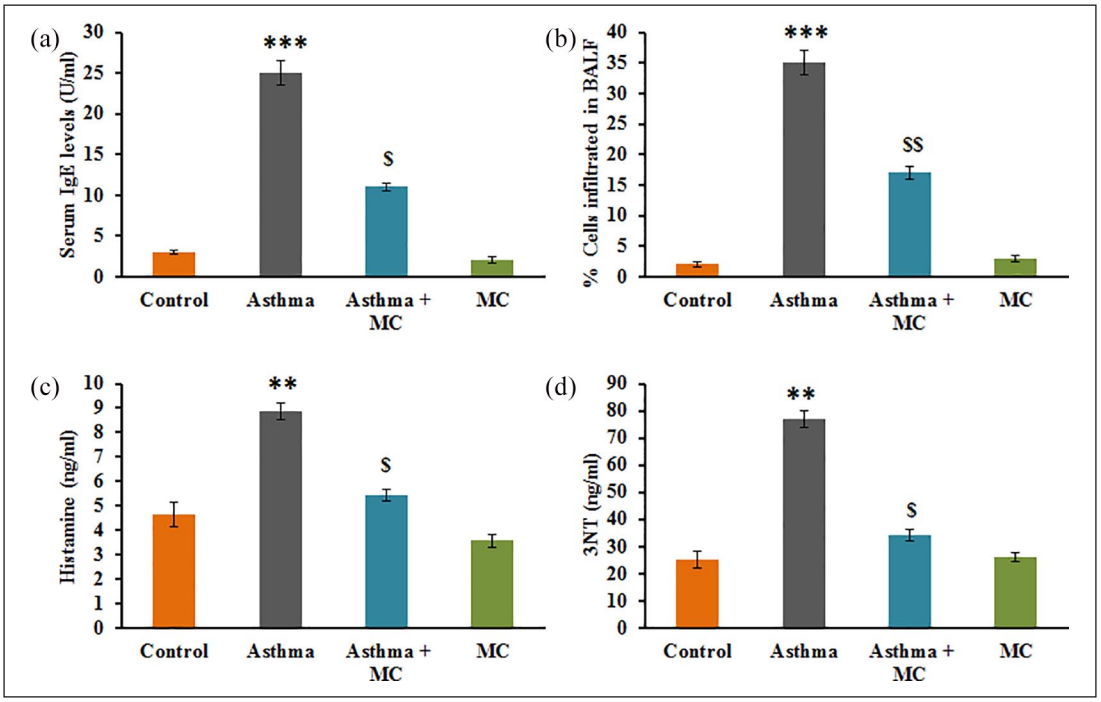

Figure 2. (a–d) IgE, infiltrating cells in BALF, histamine and nitrotyrosine levels of BALF in the control, and experimental group of rats. The experimental details were given in the methodology section. Values are expressed as mean ± SE (n = 10). Statistical significance expressed as **$P < 0.01$, ***$P < 0.001$ compared to vehicle-treated controls, $$P < 0.05$, $$$P < 0.01$ compared to asthma rats.

to control. While these inflammatory cytokines were reduced in MC treatment, demonstrate the protective effect of MC is also thorough, the modulation of inflammatory molecules (Figure 3).

Additionally, the analysis of the level of the fibrotic marker in the control and experimental of rats is presented in Figure 3. The results displayed that the asthma rats established a significant increase

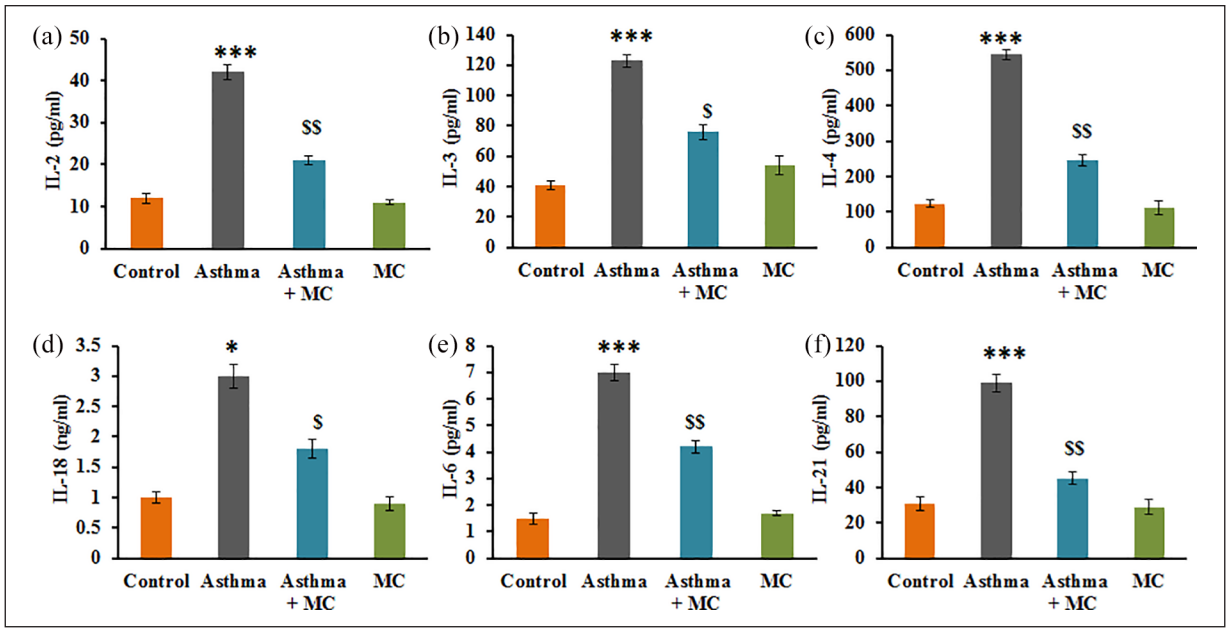

Figure 3. (a–f) Cytokine expression analysis of IL-2, IL-3, IL-4, IL-18, IL-6, and IL-21 in the control and experimental group of rats. The experimental details were given in the methodology section. Values are expressed as mean ± SE (n = 8). Statistical significance expressed as $^{*}P < 0.05$, $^{***}P < 0.001$ compared to vehicle-treated controls, $\$P < 0.05$ compared to asthma rats.

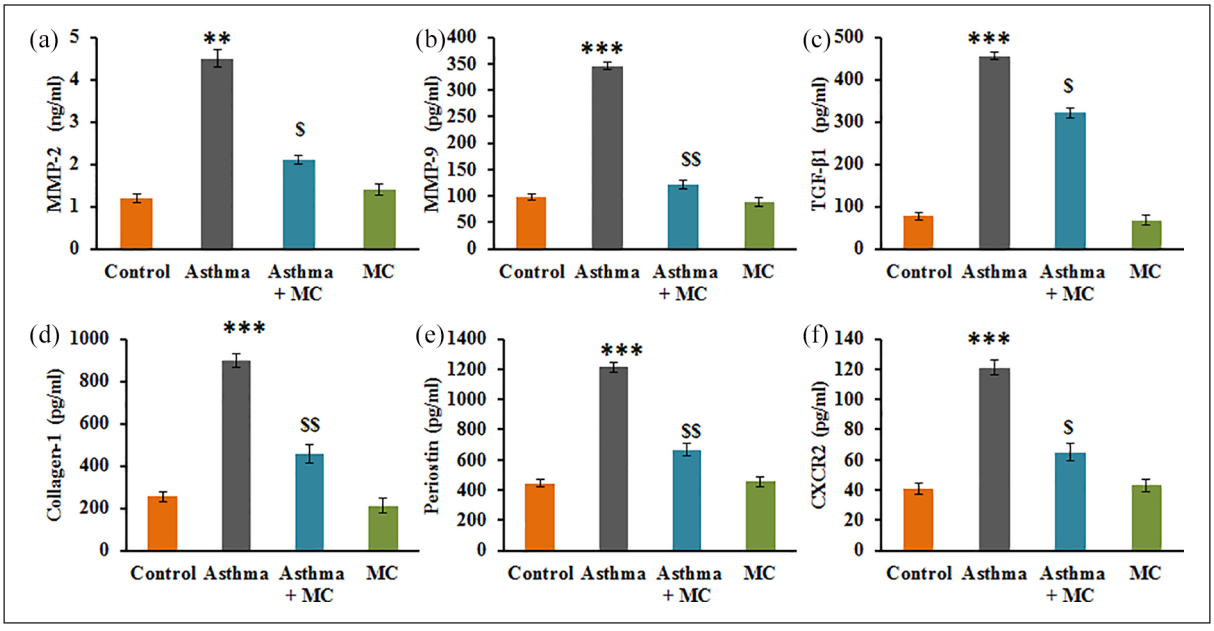

Figure 4. (a–f) The fibrosis marker expression analysis in the lung tissues of the control and experimental group of rats. The experimental details were given in the methodology section. Values are expressed as mean ± SE (n = 8). Statistical significance expressed as $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated controls, $\$P < 0.05$, $\$\$P < 0.01$ compared to asthma rats.

in the levels of matrix and fibrotic markers such as MMP-2 ($P < 0.01$), MMP-9 ($P < 0.001$), TGF-β1 ($P < 0.001$), collagen-1 ($P < 0.001$), periostin ($P < 0.01$), CXCR2 ($P < 0.05$) compared to control. While these markers were found reduced in MC treatment, suggest that the protective effect of MC is also thorough, the modulation of fibrosis-related molecules (Figure 4).

To substantiate the role of MC on the modulation of asthma-related matrix protein markers, the

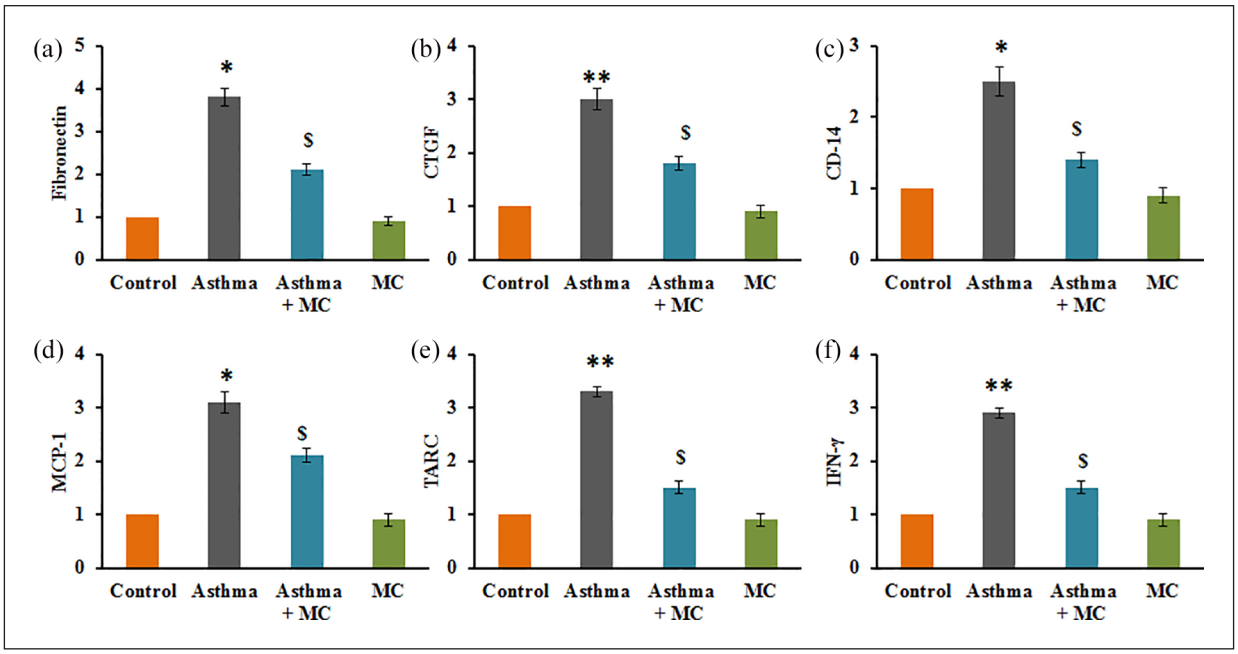

Figure 5. (a–f) qRT-PCR mRNA expression analysis in the control and experimental group of rats. The qRT-PCR experimental details were given in the methodology section. Values are expressed as mean ± SE (n = 8). Statistical significance expressed as $^{*}P < 0.05$, $^{**}P < 0.01$ compared to vehicle-treated controls, $\$P < 0.05$ compared to asthma rats.

mRNA levels of few genes were elucidated in the control and experimental of rats, and the results were presented in Figure 5. The results demonstrated that a profound increase ($P < 0.01$) in the mRNA transcript expression of fibronectin (4-fold), CTGF (3.2-fold), CD-14 (2.6-fold), MCP-1 (3.3-fold), TARC (3.2-fold), IFN-γ (2.8-fold) in rats with asthma compared to vehicle-treated controls. However, the increased levels of these marker genes were attenuated in MC treatment indicate the protective effect of MC against neonatal asthma is through the modulation of matrix-associated proteins (Figure 5).

Discussion

Asthma is a chronic inflammatory disease characterized by an increased airway hyper-responsiveness to irritants such as dust, smoke, pollen, and other allergens, causing inflammation in the airway.[19] This would cause airway tissue destruction that ultimately leads to airway remodeling that is displayed as structural changes in the airway. Symptoms of asthma include chest discomfort, mucus accumulation in the lungs, wheezing, and difficulty in breathing in human beings. However, the scientific research deployed the use of murine models of asthma that can give detailed information on the association between the (Immuno) pathology and various aspects of airway functions similar to human airways[20] during the allergen invade. These allergens activate the immune cells, and they cause an increase in the mucus secretion in the lungs and an increased infiltration of eosinophils, mast cells in the airway.[21,22]

Immune cells secrete various inflammatory cytokines, chemokines, metalloproteases that are the precursors for the onset of structural alteration in the airway that is developed into the remodeling of the airway.[22,23] These include the thickening of the basement membrane, loss of epithelial integrity, and tissue fibrosis in the sub-epithelial region. The goblet cells are enlarged and are increased in their numbers with the cartilage integrity being compromised and a large release of matrix proteins is observed asthma.[24] The deposition of the collagen in the airway wall of the animals induced with asthma with an allergen was characterized using lung histology analysis indicate the structural changes that occur in the airway wall which is part of the remodeling process.[25,26] The amount of collagen deposition indicates the severity of the disease[27] and the rigidity and the stiffness of the airway wall[28] of the affected animals. When the

asthma-induced animals were treated with MC, collagen deposition was reduced and the smooth muscle matrix indicating the amelioration of the symptoms associated with OVA-induced asthma.

The main pathological feature of OVA-induced asthma is the infiltration of the immune cells into the airway, especially the eosinophilic infiltration observed in the BALF of the OVA-induced animals. These cause the subsequent airway inflammation and elicit the hyperimmune reaction to the OVA in the induced animals. This is observed with a profound increase in the OVA-specific IgE,[29] and such increases were reversed with the treatment with MC, which has decreased the immune reactivity against OVA and decreased the IgE levels.[30] The eosinophilic infiltration has also decreased with MC treatment indicating that MC could reliable to be used against inflammation.[14] Histamine secretion is part of the hyperresponsiveness[13] and increase in nitrotyrosine due to oxidative stress[31] were also reduced with the MC treatment indicates that the effect of the drug is through alleviation of oxidative stress.

In response to the OVA stimulation, the level of IL-2 was increased significantly would have lead to T lymphocyte proliferation and production of other cytokines,[32,33] and mediators for eosinophil infiltration[33] and bronchoconstriction. Similarly, the levels of IL-4 which were elevated in asthmatic animals got reduced with MC treatment speculates that the proinflammatory cytokine-IL-6 is increased with asthmatic animals has decreased and it is attributed to the anti-inflammatory effect of MC proving its protective effect.[14,34,35] The Th2 immunity that is activated with the OVA-induction has led to increasing in IL-3[36,37] that was countered by MC. Further, IL-18, IL-21 was found increased in the serum of patients also in the asthma-induced animals induces the major role as a cofactor in Th2 cell development and is the main component in the pathogenesis of asthma.[38]

Fibrosis in asthmatic individuals is majorly contributed by MMP-9 by mediating the matrix reorganization, airway inflammation by enhancing the infiltration of eosinophils, revascularization[39] and smooth muscle hyperplasia.[40] Our results have shown that MMP-2 and MMP-9 have increased in the OVA-induced asthmatic rats and such increases have contributed to the ECM degradation and thus resulted in vascular remodeling.[41,42] Growth factors play significantly in the pathogenesis of matrix remodeling in asthma. CTGF, which plays a coordinative role in MMP-9 to increase its proteolytic role in vascular remodeling, has been found increased in asthmatic animals.[43,44] It induces the smooth muscle cells to proliferate faster and release fibronectin and collagen I in asthmatic animals.[45–47] Further, it mediates the increase of TGF-β1 to cause angiogenesis in the smooth muscle cells in the airway remodeling in OVA-induced asthmatic animals.[48] However, these effects were reduced with MC treatment which is already substantiated in other inflammatory diseases.[12]

High expression levels of periostin have been observed in in vivo models of asthma[49] having high angiogenesis, and TGF-beta1 expression was observed in the present study asthmatic rat model. It is well known for its association with sinus[50] and severe inflammatory conditions of fibrotic remodeling.[51] Severe exacerbations of asthma occur with the neutrophil recruitment to the lung tissue on the increased expression of CXCR2 that attracts to its ligand CXCL8.[52–54] the present study observation of an elevated CD14 in asthma affected animals suggest that the disease severity due to OVA is on the next stage and it involves macrophage activation and hence the observation,[55] and it can be reversed with the anti-inflammatory action of MC.[56,57] Other inflammatory mediators such as MCP-1,[58] TARC and IFN-gamma[34] were also reduced with the anti-inflammatory action of MC.

Conclusion

Hence, in our findings, it was clearly stated that the airway remodeling occurs with the eosinophil infiltration into the smooth muscle cells, which was augmented, by growth factors. These effects were high in the OVA-induced asthmatic animals, and these effects were controlled by the administration of MC that reduced the effects of inflammation in the airway and the subsequent structural alterations in the process of airway remodeling. Our candidate molecule could be an effective drug in the fight against asthma-related complications without having any known side effects. We have a disadvantage that our results did not state the pathway or the molecular mechanism that is involved in the drug actions against asthma. Hence, it could be asserted in the later research that would be useful in the development of its drug. Even though the present research demonstrated the protective efficacy of MC in a

small animal model, the study has its own limitations with respect to the extent to which they accurate human disease. Hence, an extensive validation by clinical studies in the future is warranted.

Author contributions

Y.D., J.L., R.P.J., J.L. carried out all experiments; Y.M. designed the experiments and wrote the manuscript. All authors read and approved the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

All procedures involving animals in this study confirmed to the Regulations for the Administration of Affairs Concerning Experimental Animals and were under the approval of the Institutional Animal Ethical Committee of No. 115, Shandong Provincial Third Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250031, Shandong, China. All efforts were made to minimize the suffering of the animals.

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