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Tiron ameliorates oxidative stress and inflammation in a murine model of airway remodeling

Nagla A. El-Sherbeeny [a,*], Zeinab A. Hassan [b], Hayam Ateyya [c]

[a] Department of Clinical Pharmacology, Faculty of Medicine, Suez canal University, Egypt
[b] Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Egypt
[c] Department of Clinical Pharmacology, Faculty of Medicine, Cairo University, Egypt

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ABSTRACT

Airway remodeling includes lung structural changes that have a role in the irreversibility of pulmonary dysfunction shown in chronic bronchial asthmatics. The current experiment investigated the effect of the mitochondrial antioxidant, tiron in comparison with dexamethasone (DEXA) on airway remodeling in chronic asthma. Sensitized BALB/c mice were challenged with ovalbumin (OVA) aerosol for 8 weeks, OVA sensitized-challenged mice were treated with either DEXA or tiron, respectively. After that, lung tissue and bronchoaveolar lavage fluid (BALF) were used for measurement of different biological markers. Lungs were examined for histopathological changes and immunohistochemistry. Upon comparing with vehicle treated animals, trion or DEXA treatment significantly reduced eosinophils, lymphocytes, neutrophils and macrophages count in the BALF. Both drugs significantly alleviated chronic OVA-induced oxidative stress as illustrated by decreased pulmonary malondialdenhyde (MDA) and increased glutathione (GSH) and superoxide dismutase (SOD) levels. Asthmatic mice exhibited elevated levels of NOx, IL-13 and TGF-β1 that were reduced by DEXA and tiron. Histopathological changes and increased immunoreactivity of nuclear factor-Kappa B (NF-κ B) in OVA-challenged mice were minimized by tiron and DEXA treatment. In conclusion, in this model of chronic asthma DEXA and tiron ameliorated airway remodeling and inflammation in experimental chronic asthma with no difference between the effect of tiron and DEXA. Tiron has a potential role as adjuvant treatment in chronic asthma.

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1. Introdution

Bronchial asthma is manifested by repeated reversible episodic airway obstruction with an ongoing process of airway inflammation [1]. Asthma occurs as a consequence of genetic and environmental factors interaction such as a family history of allergic reaction, the presence of immunoglobulin E (IgE) which is allergen-specific, viral respiratory diseases, exposure to aeroallergens such as dust mites, mold spores, seasonal pollen, animal allergens, and nonallergens such as smoke, odors, cold air, chemicals, medications, exercise, obesity, and lower socioeconomic status [2].

Airway wall remodeling and airway hyperresponsiveness are two prominent features of asthma that contribute to chronic symptoms. Remodeling of airway is characterized by a number of structural changes, including extracellular matrix component deposition like collagen, laminin, fibronectin, and tenascin, airway smooth muscle hypertrophy and hyperplasia of both muscle and goblet cells. Airway remodeling is one of the causes leading to a decrease in the effectiveness of standard therapy [3]. Airway hyperresponsiveness is a complicated process, and several factors participate in its progress. Inflammation of mucosal airway is an influx of eosinophils, activated T lymphocytes with release of Th2 cytokines (particularly IL-4, IL-5, and IL-13), eotaxin, RANTES (Regulated on Activation, Normal T Expressed and Secreted) and other proinflammatory mediators which play crucial factor in regulating the allergic inflammatory reaction that progress to airway hyperresponsiveness [4,5].

Numerous investigation have shown that airway inflammation is the principal reason for frequent attacks of airway obstruction in asthmatic patients and proved that reactive oxygen species (ROS) act a predominant function in starting and intensifying inflammation in airways of asthmatic [6]. These ROS are produced from airways of asthmatic recruited inflammatory cells. There are many antioxidants acting to overcome ROS-induced damage of several cellular molecules such as lipid membranes, proteins, and DNA [1]. On the other hand, during asthma there is an excessive production of ROS that may overwhelm these defenses causing inflammatory changes in the airways and systemic circulation. ROS-induced injury may lead to enhanced smooth muscle contraction, vascular permeability and mucus hypersecretion [7].

Designs aiming to increase endogenous antioxidants via dietary or pharmacological interference in order to restore the oxidant/

* Corresponding author at: Department of Clinical Pharmacology, Faculty of Medicine, University Road, Ismailia, Egypt.
E-mail address: n_elsherbeeny@hotmail.com (N.A. El-Sherbeeny).

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antioxidant balance in asthma are the modern region of investigation all over the world.

Tiron (4, 5-dihydroxy-1,3-benzene disulfonic acid) is a water-soluble cell permeable analog of vitamin E and function as a direct hydroxyl radical and superoxide scavenger [8,9]. It is both a beneficial protective antioxidant and an efficient nontoxic metal chelator that has been commonly used in oxidative stress-associated physiologic studies [10].

The utilization of tiron as antioxidant may introduce an additional remedial modality for controlling allergic asthma. Thus, this research aims at providing the role of tiron in boosting the endogenous antioxidants or scavenging excessive ROS production and examining if it can dampen/prevent the inflammatory response. The study compares the effect of tiron with the standard antiasthmatic medication DEXA in chronic asthmatic mice.

2. Material & methods

2.1. Animals

BALB/c female mice aged approximately 8 weeks were purchased from the National Research Centre, Dokki, Giza, Egypt. During the time of the experiment, mice were housed under pathogen-free conditions. Animals were kept in a room with standard controlled temperature and a 12-h dark/light cycles, and permitted free access to food and water. The experimental protocol of this study followed carefully the ethical guidelines and the principals of care, use and handling of experimental animals approved by "The research Ethics Committee", Faculty of medicine, Suez Canal University and Faculty of medicine, Cairo University, Egypt, which are in agreement with "Principles of Laboratory Animals Care" (NIH publication No. 85-23, revised 1985).

2.2. Protocol for sensitization and antigen challenge

Mice were allowed to adapt for the week preceding the beginning of the study. On days 0 and 14 sensitization of animals was achieved by intraperitoneal injection of 10 μg OVA emulsified in 500 μg of aluminium hydroxide in 200 μl saline. At 21 day, mice were exposed to OVA aerosol (1% weight/volume diluted in sterile physiological saline) for 30 min and the challenge repeated three times a week for 8 weeks. Aerosol was generated using an ultrasonic air nebulizer (Omron, Vernon Hills) attached to a cylindrical chamber (20 × 30 × 35 cm). The outlet of the chamber was connected to a vacuum pump with the flow maintained at a rate of 15 ml/min and particle size 2–6 μm adjusted by a flow meter (Hoffer Flow Controls, USA). Mice were placed in the chamber and exposed to the aerosol for 30 min during each designated challenge session.

2.3. Experimental groups

72 mice were randomly divided into groups (12 mice each):

Group I (normal group): saline treated mice.

Group II (control group): Mice alum sensitized and saline challenged. Mice received 0.5% CMC (10 ml/kg/day, orally) on the day of challenge, the dose was given 1 h before each challenge.

Group III (Tiron-control): mice alum sensitized, saline challenged and received 200 mg/kg tiron daily by gavage.

Group IV (asthmatic mice): mice OVA sensitized and challenged.

Group V (DEXA-OVA): asthmatic mice treated by daily I.P. injection of 2 mg/kg DEXA.

Group VI (Tiron-OVA): asthmatic mice given tiron.

The daily doses of treatment drugs were given 1 h before the challenge.

Twenty-four hours after the final challenge, mice were anaesthetized using I.P. urethane (2.5 mg/kg). To collect BALF for subsequent analysis, the chest of each mouse was opened, the trachea with the heart-lung package was exposed and the left main bronchus clamped. A cannula was carefully inserted into the trachea, and the right lung was lavaged three times using 1 ml PBS solution. The inferior lobe of the left lung was dissected and homogenized while the remaining part of left lung was collected for histopathology.

2.4. BALF preparation and white blood cell count

Centrifugation of BALF was carried out for 10 min at 1000 g and 4 °C. The pellet of cells was redistributed in fresh PBS (100 μl) and the supernatant was cautiously taken away and kept at −80 °C. The total number of inflammatory cells in BALF was counted using a hemocytometer. For differential white blood cell count, smears of BALF were prepared then stained with Diff-Quik solution (Dade Behring Inc., Newark, NJ, USA). Four different random locations at 40 magnifications were examined and at least 200 cells were counted in each one.

2.5. Lung homogenate preparation

The lung slices were weighed then homogenized (1:10, w/v) in 0.1 M phosphate buffer (pH 7.4) in an ice bath, then subjected to centrifugation for 20 min (3000g, 4 °C). The supernatant obtained was utilized for evaluation of the lipid peroxidation marker, MDA and antioxidants SOD and GSH concentrations.

2.6. Estimation of lipid peroxidation and antioxidants in lung tissue

Malondialdehyde (MDA) [11], superoxide dismutase (SOD) [12] and reduced glutathione (GSH) amount [13] in lung homogenates were measured (Bio-diagnostic Company, Giza, Egypt).

2.7. Estimation of nitric oxide in BALF

As an indication of nitric oxide production, the total production of nitrate/nitrite (NOx) was evaluated in BALF using NO assay kit (R&D Systems, Minneapolis, USA).

2.8. Measurement of IL-13 & TGF-β1 levels in BALF

Cytokines IL-13 and TGF-β1 concentration in BALF was determined via DuoSet ELISA kit for mouse interleukin-13 (Catalog Numbers: DY413-05 and Quantikine ELISA for TGF-β1 (Catalog Number MB100B, R&D Systems).

2.9. Tissue histopathology and immunohistochemistry

2.9.1. Histopathology of the lung

The left lungs were carefully dissected from mice. Immediately after dissection, the lungs were inflated with the fixation solution consisting of 10% neutral buffered formalin by insertion of 5 ml syringe through the opening of the trachea [14]. Sections were dehydrated and embedded in paraffin. Lung sections were sliced at 5 μm thickness and stained with hematoxylin and eosin (H&E) to determine the airway inflammatory cell infiltrate [15], Periodic Acid -Schiff (PAS), to identify goblet cells [15] or Masson's trichrome (MT) stain to determine collagen deposition/fibrosis [16]. All histological examinations were carried out by an observer blinded to the design groups.

2.9.2. Immunohistochemistry (IHC) for NF-κB expression

Lung samples were treated in 4% paraformaldehyde and rooted in paraffin wax then sectioned at 5 μm for IHC. Immunostaining was carried out via Avidin-Biotin complex (ABC) method [17]. After preparing the tissue slides, blocking was done using endogenous peroxidase with 3% H2O2. Next, the slides were incubated for the night at 4 °C with a rabbit polyclonal antibody directed against NF-κB p65 in a dilution of 1:100 (purchased from Santa Cruz Biotechnology, Santa Cruz,

CA, USA). The sections then washed with PBS followed by incubation with biotinylated anti-mouse IgG (purchased from Sigma Chemicals Co., St Louis, MO, USA). After that, the slides were incubated with a solution of Avidin–Biotin–Peroxidase complex. Visualization was performed using 0.05% (w/v) 3, 3′-diaminobenzidine tetrahydrochloride. The slides were counterstained by Mayer's Hematoxylin. Positive reaction emerges as a brown color distinguished both in the cytoplasm and nuclei. Positivity was estimated only by nuclear immunostaining. Leica Qwin 500 imaging System was used for counting NF-κB immunopositive cells [18].

2.10. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical testing for parametric results were done via one-way analysis of variance (ANOVA) followed by post hoc Dunette test for selected pair of groups. Non-parametric testing were done via Kruskal–Wallis test followed by Dunn's post-test correction. All tests were performed using Instat-2 program (GraphPad Software Inc. V2.04, San Diego, CA, USA).

3. Results

Tiron-control group exhibited no notable alteration in investigated parameters compared to control and normal groups. In addition, the statistical analysis revealed no significant variation among the OVA-challenged groups treated by DEXA and tiron respectively in all measured parameters.

3.1. Effect of tiron on BALF cell count

Challenged mice exhibited significant elevation in total and differential cell counts after long-term OVA inhalation compared to the controls (Table.1). The total and differential inflammatory cell counts were reduced by tiron and DEXA treatment in OVA challenged animals.

3.2. Effect of tiron on oxidative stress in lung tissue

Mice with chronic asthma showed marked increase in oxidative stress in lung tissues that is evident by significant increased MDA and a marked decline in both GSH and SOD concentration compared to controls (Table 2). The increased MDA level in the lung of mice was notably ameliorated upon DEXA and tiron treatment. Also a significant elevation in both GSH and SOD levels were observed in the lung tissues of both DEXA and tiron treated mice compared to asthmatic mice. DEXA or tiron treated-mice ameliorated considerably chronic OVA-induced oxidative stress.

Table 2
Effect of tiron on oxidative damage in lung tissue of chronic asthmatic mice.

	Oxidative stress parameter in lung tissue		
	MDA (nmol/g tissue)	GSH (μmol/g tissue)	SOD (unit/g tissue)
Control	35 ± 2.9	8.71 ± 0.77	9.45 ± 0.9
Tiron-saline	37 ± 2.7[a]	8.53 ± 0.66[a]	9.57 ± 0.72[a]
OVA	75.5 ± 7.3[b]	2.99 ± 0.25[b]	2.85 ± 0.31[b]
OVA-DEXA	43 ± 4.43[a]	7.53 ± 0.45[a]	8.24 ± 0.65[a]
OVA-tiron	47 ± 4.72[a]	7.01 ± 0.55[a]	7.3 ± 0.73[a]

Data were expressed as mean ± SEM, n = 12.
OVA challenge was performed for 30 min after sensitization 3 times a week for 8 weeks and the drugs were administered 1 h before OVA challenge.
OVA; ovalbumin, DEXA; dexamethasone, MDA; malondialdehyde, GSH; glutathione; SOD; superoxide dismutase.
[a] Significantly different from their corresponding OVA-challenged groups (one-way ANOVA test followed by Dunette post hoc test at ($p < 0.05$).
[b] Significantly different from their corresponding control.

3.3. Effect of tiron on NOx, IL-13 & TGF-β1 in BALF

Asthmatic mice exhibited statistically significant higher levels of NOx, IL-3 and TGF-β1 production upon comparing with control group, and this OVA-exacerbation of NO and cytokines was significantly reduced by treatment with DEXA or tiron compared to asthmatic mice (Table 3).

3.4. Effect of tiron on lung histopathology

Examination of control lung and tiron-treated lung showed normal lung structure with branched thin walled blood vessels. Bronchioles were lined with pseudostratified columnar epithelium surrounded by thin circular smooth muscle fibers. Blood capillaries lie in the thin inter-alveolar septa (Fig. 1A & B, respectively). In contrast, OVA-challenged lung showed increased epithelial thickness with thickened interalveolar septa containing congested blood vessels. The leakage of different inflammatory cells surrounding bronchioles and blood vessels were also noticed (Fig. 1C & D). Lung from DEXA- and tiron-OVA challenged mice showed marked improvement of its morphological organization with minimal signs of inflammation or inflammatory cell infiltration (Fig. 1E & F, respectively).

3.5. Effect of tiron on lung scores of goblet cells and fibrosis and expression of NF-κB

Examination of control lung and tiron-treated lung showed weak PAS positive cells in the airway (Fig. 2A & B, respectively and Table 4). On the other hand, MT stained section of control lung and tiron-treated lung showed few subepetheilial collagen fibers in the bronchioles and few collagen fibers in all interalveolar septa and around blood vessels (Fig. 3A & B, respectively and Table 4). In addition,

Table 1
Effect of tiron on total and differential cell count in chronic asthmatic mice.

	Cell count in bronchoalveolar lavage fluid ($\times 10^4$/lung)				
	Total cell	Lymphocytes	Eosinophils	Neutrophils	Macrophages
Control	7.8 ± 0.93	5.05 ± 0.75	0.0 ± 0.0	2.92 ± 0.21	0.0 ± 0.0
Tiron-saline	6.9 ± 0.92[a]	4.9 ± 0.59[a]	0.0 ± 0.0[a]	2.1 ± 0.33[a]	0.0 ± 0.0[a]
OVA	30.67 ± 4.51[b]	24.51 ± 2.53[b]	0.9 ± 0.09[b]	5.57 ± 0.75[b]	0.24 ± 0.02[b]
OVA-DEXA	8.57 ± 0.95[a]	5.55 ± 0.69[a]	0.0 ± 0.0[a]	3.22 ± 0.34[a]	0.0 ± 0.0[a]
OVA-tiron	10.84 ± 1.27[a]	7.07 ± 0.85[a]	0.05 ± 0.01[a]	2.34 ± 0.33[a]	0.0 ± 0.0[a]

Data were expressed as mean ± SEM, n = 12.
OVA challenge was performed for 30 min after sensitization 3 times a week for 8 weeks and the drugs were administered 1 h before OVA challenge.
OVA; ovalbumin, DEXA; dexamethasone.
[a] Significantly different from their corresponding OVA-challenged groups (one-way ANOVA test followed by Dunette post hoc test at ($p < 0.05$).
[b] Significantly different from their corresponding control.

Table 3
Effect of tiron on NOx, IL-13 & TGF-β1 in BALF of chronic asthmatic mice.

	Parameter in BALF		
	NOx (μmol/ml)	IL-13 (pg/ml)	TGF-β1 (pg/ml)
Control	11.5 ± 0.97	20 ± 0.19	5.67 ± 0.45
Tiron-saline	10.47 ± 0.95[a]	18.7 ± 1.7[a]	6.47 ± 0.61[a]
OVA	27.95 ± 2.51[b]	125.5 ± 9.57[b]	14.91 ± 0.99[b]
OVA-DEXA	9.85 ± 0.75[a]	35.57 ± 3.22[a]	7.07 ± 0.75[a]
OVA-tiron	8.25 ± 0.8[a]	45.87 ± 4.39[a]	9.25 ± 0.83[a]

Data were expressed as mean ± SEM, n = 12.
OVA challenge was performed for 30 min after sensitization 3 times a week for 8 weeks and the drugs were administered 1 h before OVA challenge.
BALF; bronchoalveolar lavage fluid, OVA; ovalbumin, DEXA; dexamethasone, TGF- β1; transforming growth factor-beta 1, NOx; nitrate/nitrite, IL-13; interleukin-13.
[a] Significantly different from their corresponding OVA-challenged groups (one-way ANOVA test followed by Dunette post hoc test at ($p < 0.05$).
[b] Significantly different from their corresponding control.

immunohistochemical examination of the same groups showed few epithelial cells showing nuclear immunoreactivity (Fig. 4A & B, respectively and Table 4).

Examination of OVA-challenged lung showed more PAS positive cells and mucous hypersecretion within the bronchioles in the lung indicating hyperplasia of goblet cells compared with the control group (Fig. 2C & D, respectively). Results also showed that lungs of the OVA group had a significant elevation of subepithelial fibrosis in the bronchioles and moderate aggregations of collagen fibers in the thickened interalveolar septa (Fig. 3C) and significant increase in immune-staining activity of NF-κB compared with control (Fig. 4C & Table 4 respectively).

Examination of both DEXA and tiron treatment groups showed a significant decrease in goblet cell hyperplasia and mucus secretion compared with OVA group (Fig. 2E & F respectively). Both treatments also precluded the airway epithelium thickening progression observed in mice of OVA challenged groups with relatively few subepithelial collagen fibers in the bronchioles and most of the interalveolar septa (Fig. 3D & E respectively & Table 4). Administration of both DEXA and tiron also decreased the number of immune-stained cells compared to ova challenged mice indicating its effective antioxidant effect through affecting NF-κB expression, however there was still mild immune-stained cells compared to normal group (Fig. 4E & F & Table 4).

4. Discussion

In the current research chronic experimental asthma in mice that was previously shown to simulate the characteristic features of the human asthma [19] was used to explore the effect of the antioxidant,

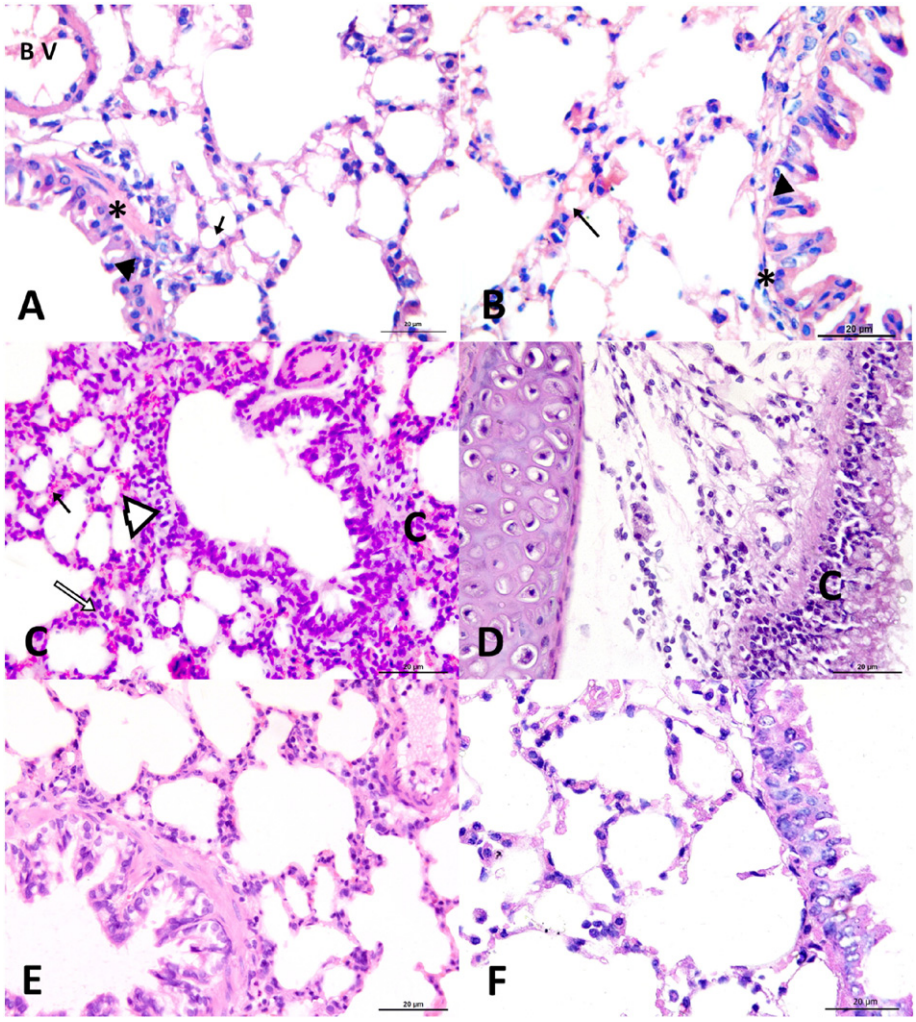

Fig. 1. Effect of DEXA and tiron on lung inflammation in chronic murine asthma (H & E stain, A–C & E scale bar; 20 μm and D scale bar; 50 μm). A) Control lung and B) tiron-treated lung showed normal lung structure with branches of thin walled blood vessels. Bronchioles are lined with pseudostratified columnar epithelium (arrow head) surrounded by thin circular smooth muscle fibers (asterisk). Blood capillaries lie in the thin inter-alveolar septa (arrow). C & D) OVA-challenged lung showed increased epithelial thickness with thickened interalveolar septa containing congested blood vessels (white arrows) and many inflammatory cells (white arrow heads). The infiltration of various inflammatory cells (C) around and bronchioles are also noticed. E) DEXA-OVA challenged lung and F) tiron-OVA challenged lung shows marked improvement of its morphological organization.

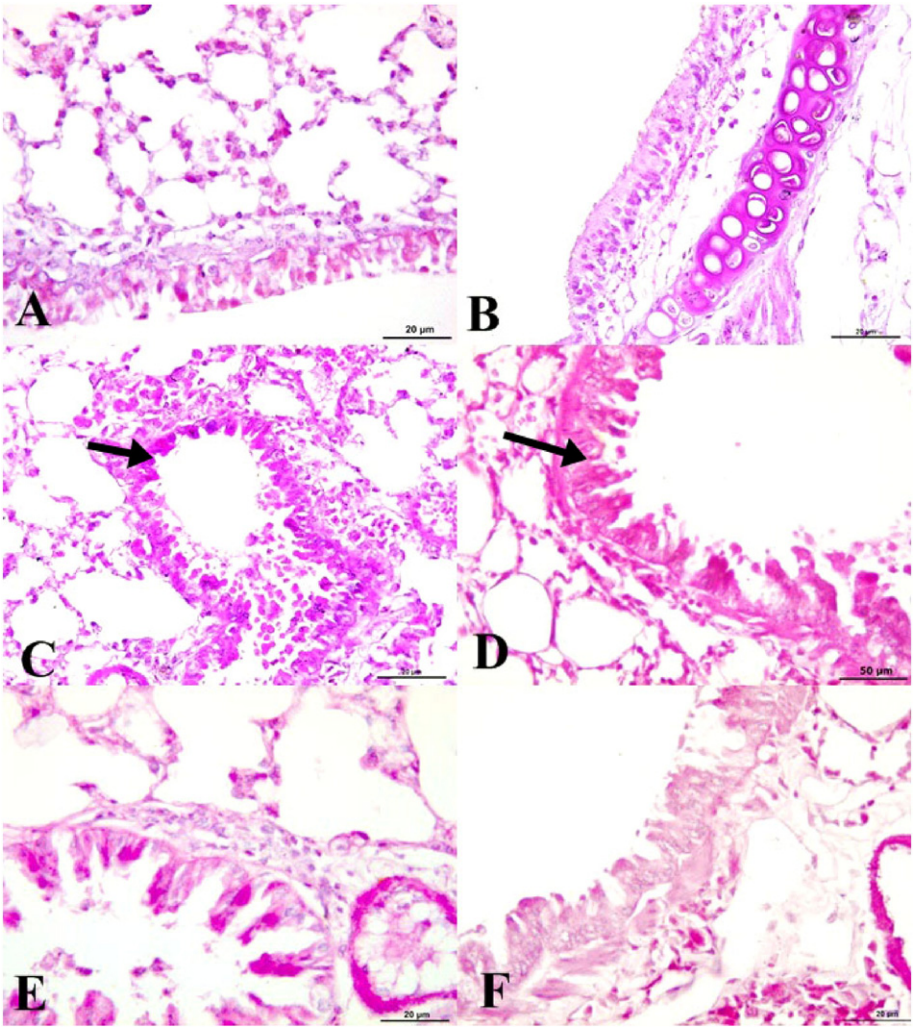

Fig. 2. Effect of DEXA and tiron on scores of lung goblet cells in chronic murine asthma (PAS stain, scale bar; 20 µm). A) Control lung and B) tiron-treated lung shows weak PAS positive cells in the airway. C) & D) OVA-challenged lung showed more PAS positive cells and mucous hypersecretion indicating the goblet cell hyperplasia. E) DEXA-OVA challenged lung and F) tiron-OVA challenged lung shows marked improvement with minimal goblet cell hyperplasia.

tiron on airway remodeling in comparison with the antiasthmatic glucocorticoid, dexamethasone. Suppression of airway remodeling by glucocorticoid treatment is well authenticated in human and in experimental animals [20–22]. Results showed a non-considerable discrepancy between the effect of DEXA and tiron. Both tiron and DEXA were able to reduce airway oxidative stress, inflammation and remodeling in asthmatic mice. DEXA or tiron treatment caused a notable reduction in both total and differential white blood cell count induced by OVA in BALF.

Table 4
Effect of tiron on lung goblet cell score, fibrosis score and NF-κB expression in chronic asthmatic mice.

	Parameter		
	Fibrosis score	Goblet cell score	NF-κB score
Control	0.0 ± 0.0	0.0 ± 0.0	12 ± 0.16
Tiron-saline	0.0 ± 0.0	0.0 ± 0.0	15.98 ± 1.7
OVA	3.7 ± 0.15[a]	3.8 ± 0.22[a]	49.56 ± 5.2[a]
OVA-DEXA	1.6 ± 0.22[a,b]	1.4 ± 0.22[a,b]	25.09 ± 2.6[a,b]
OVA-tiron	1.8 ± 0.25[a,b]	2 ± 0.15[a,b]	22.98 ± 2.02[a,b]

Data were expressed as mean ± SEM, n = 12.
OVA challenge was performed for 30 min after sensitization 3 times a week for 8 weeks and the drugs were administered 1 h before OVA challenge.
OVA; ovalbumin, DEXA; dexamethasone, NF-κB, nuclear factor kappa B.
[a] Significantly different from their corresponding control group.
[b] Significantly different from their corresponding OVA-challenged group (Kruskal–Wallis test followed by Dunn's multiple comparisons test, significance at ($p < 0.05$).

OVA-challenged lung showed histopathological changes with increased epithelial thickness, thickened interalveolar septa containing congested blood vessels, and leakage of various inflammatory cells around bronchioles and blood vessels with engorged blood vessels and cellular leakage in OVA-challenged lung group, indicating the occurrence of inflammatory reactions. Capillary congestion in the same group might also have resulted from the formerly mentioned inflammatory reactions or owing to the chemical mediators liberated from alveolar macrophages after tissue injury [23]. Treatment with tiron or DEXA in OVA challenged mice resulted in marked improvement of lung morphological organization with minimal inflammatory cell leakage, reversal of goblet cell hyperplasia and subepithelial deposition of collagen.

In the current study, chronic 8 weeks challenge with OVA produced notable change in total and differential leucocytes count compared to the corresponding control groups. Consequently in 8-week challenged group, both tiron and DEXA exhibited a marked decline in total and differential cell counts upon comparing with asthmatic groups. Nader [24] reported similar results regarding BALF cell count. Also, Foster et al. [25] found significant increased total and differential cell count in BALF in models of both acute and subacute asthma but did not found the same elevation in chronic asthma. The low levels of eosinophils and

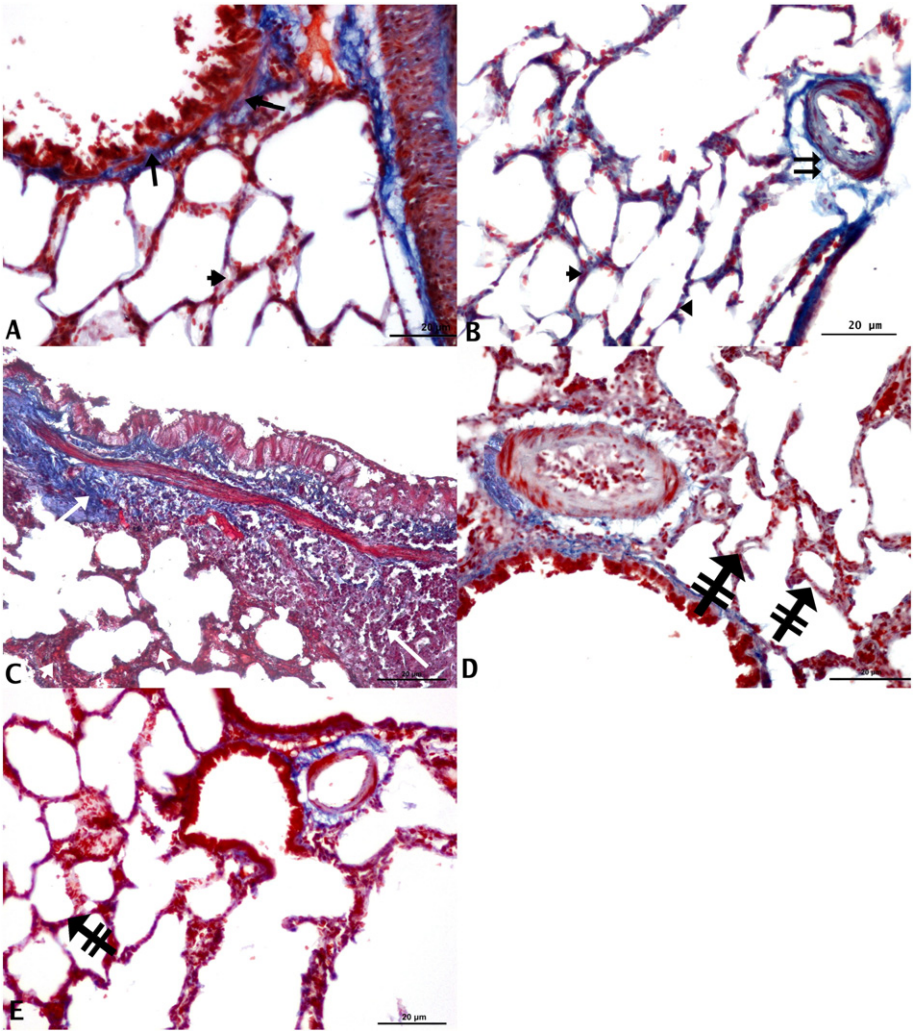

Fig. 3. Effect of DEXA and tiron on lung fibrosis in chronic murine asthma (Masson's trichrome, scale bar; 20 µm). A) Control lung and B) tiron-treated lung shows few subepetheilial collagen fibers (arrow) in the bronchioles and few collagen fibers (arrow head) in all interalveolar septa and around blood vessels (double arrows), C) OVA-challenged lung, showing many subepithelial collagen fibers (white arrow) in the bronchioles and moderate aggregations of collagen fibers (white arrow head) in the thickened interalveolar septa D) DEXA-OVA challenged lung and E) tiron-OVA challenged lung shows relatively few subepithelial collagen fibers (cross arrow) in the bronchioles and most of the interalveolar septa.

lymphocytes in BALF in chronic allergen challenge were also announced by Wegmann et al. [26] and Southam et al. [27]. A diminution in inflammatory BALF cells with continuation of lung tissue inflammation was considered as a signal of poor consequence in asthma [28]. Alrifai et al. [29] proved that in chronic asthma, maintenance of the disease is associated with persistence of tissue inflammation while bronchoalveolar inflammation declines with time. These studies concluded that deterioration of chronic asthma is correlated with lowered inflammatory cell count in BALF and continuation of tissue inflammation and that these changes are independent from resolution. This difference between BALF and tissue inflammation represents a compartmentalization of inflammatory cells that appears to be crucial for the progression from the acute to the chronic phenotype [29]. Although the exact mechanism of such compartmentalization of inflammatory cells is yet to be fully explained, some mechanisms have been postulated including changes in the expression of adhesion molecules, chemokines, chemokine receptors of adhesion molecules, chemokines and/or chemokine receptors directed by selective and spatial recruitment process [29,30]. The same studies that reported this compartmentalization in chronic allergic airway disease also reported persistent goblet cell hyperplasia. The discrepancy between the results of various studies regarding BALF total and differential count have been attributed to the use of different mice strains that could point out a contribution of the genetic factors in asthma susceptibility and recovery [31,32].

To examine the possible mechanisms mediating tiron's decreased airway inflammation and remodeling in chronic asthma, oxidative stress/antioxidant, fibrotic and inflammatory markers were measured either in BALF or lung tissue. DEXA and tiron improved prooxidants/antioxidants balance, reduced NOx, inflammatory cytokines IL-13 and TGF-β1 and caused down-regulation of the transcription factor NF-κB protein expression in lung tissue induced by OVA challenge.

Airway remodeling is considered to be the consequence of disproportion between regeneration and repair mechanisms that results in structural changes of the airway. These changes include hyperplasia of epithelial lining, vascular changes, and subepithelial fibrosis [33]. An increase in BALF Th2 cytokine IL-13 was observed. Eosinophils contribution to inflammation in bronchial asthma is mediated through release of proteins from their pro-inflammatory granules, generation of lipid-derived mediators and providing TGFβ1 [34,35]. Tiron and DEXA significantly reduced eosinophilia and IL-13 levels in OVA-challenged mice. The decrease in IL-13 levels could explain the drugs effect on eosinophils as this cytokines stimulates eosinophil recruitment into lung [36].

Mechanisms mediating inhibition of remodeling by tiron is not completely clear. However, inhibition of growth factors for fibroblasts could be one of the plausible mechanisms. TGF-β1 produced by eosinophils acts a considerable function in airway remodeling through increased formation and accumulation of extracellular matrix proteins as collagens and mediating the differentiation of fibroblast into the

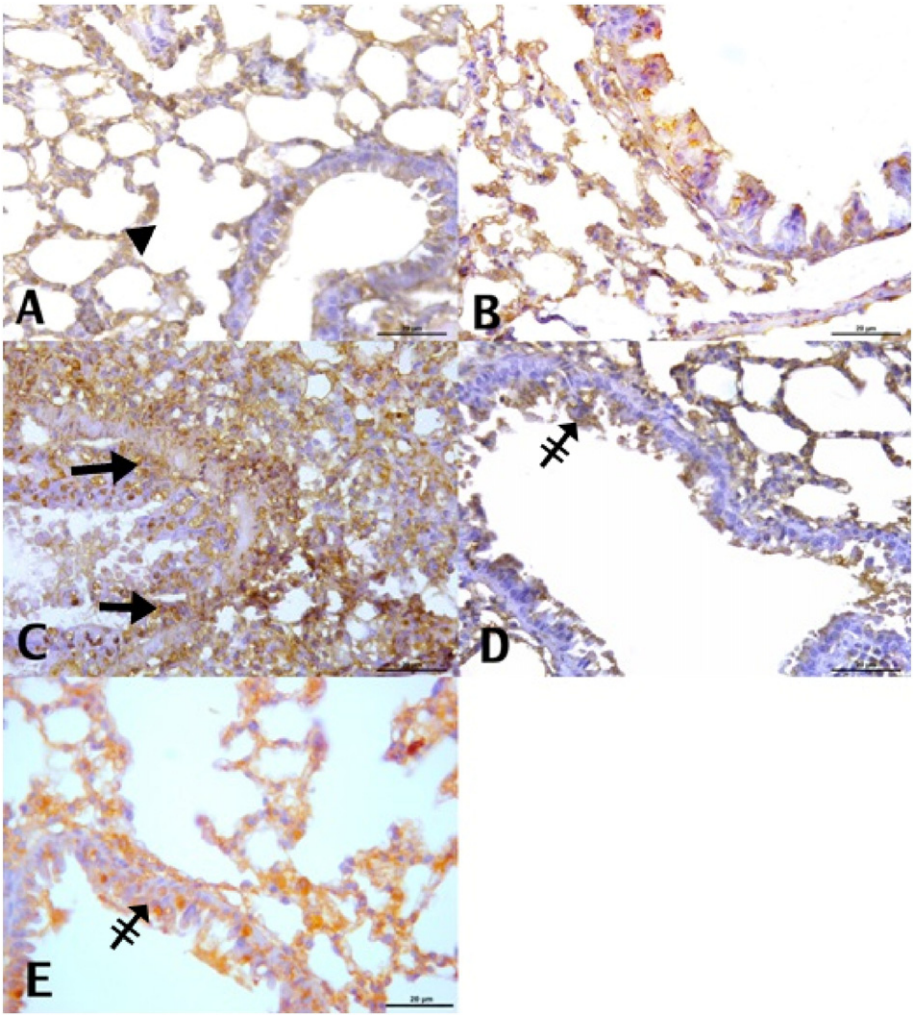

Fig. 4. Effect of DEXA and tiron on expression of nuclear factor kappa B (NF-κB) in the lung in chronic murine asthma (Avidin- biotin- peroxidase system scale bar; 20 μm). Immunoreactivity was identified as nuclear and cytoplasmic brown staining of cells. Positivity was considered only as nuclear immunostaining. A) Control lung and B) tiron-treated lung shows few epithelial cells showing nuclear immunoreactivity (arrow head). C) OVA-challenged lung showing many immunoreactive epithelial cells (arrow). D) DEXA-OVA challenged lung and E) tiron-OVA challenged lung shows less immunoreactive cells.

myofibroblast phenotype [37,38]. Based on previous research, antioxidants are considered to have a potential as one of therapeutic options for lung fibrosis [39]. The effect of tiron on TGF-β-1 was reported in a study using human renal proximal tubular HK-2 cells. Tiron blocked the overexpression of IL-6, monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and TGF-β1 induced in these cells by melamine and the authors postulated that antioxidant action of tiron played an important role in achieving this effect [40]. IL-13 and TGF-β1 perform an action in the progress of asthma and remodeling. In an experimental asthma, inhibition of IL-13 decreased airway hyperreactivity and eosinophil leakage into the airways, and suppressed the process of airway remodeling [41]. Activated eosinophils are a main source TGF-β1 [35]. Lee et al. [42] demonstrated that IL-13 could induce tissue fibrosis by selectively stimulating TGF-β1 and that the two cytokines -IL-13 and TGF-β1 together may enhance the libaration of eotaxin from human fibroblasts. Previous studies suggest an important function of TGF-β1 in airway remodeling process. TGF-β1 accelerates fibrotic changes in chronic asthma via the deposition of extracellular matrix and TGF-β1 expression was established to be related to basement membrane thickness and fibroblast number [4]. In this study, results demonstrated that the IL-13 and TGF-β1 levels in asthmatic mice were considerably elevated than those detected in normal mice while treatment with DEXA or tiron caused a significant decrease of the measured levels. Vanacker et al. [21] proved that treatment with DEXA significantly decreases subepithelial expression of TGF-β-1 in OVA-challenged mice. In the current study, results of histologic studies of DEXA and trion groups are concomitant with the other measured parameters and revealed a significant amelioration of the number of cells staining positive for mucus, collagen and fibrosis, and a decrease in the thickness of the smooth muscle layer of the airway lining compared to OVA challenged mice.

Our results revealed that the decrease in IL-13 and TGF- β1 in tiron and DEXA treated groups was accompanied by downregulation NF-κB expression. This transcription factor is considered to have a function in the progression of asthma both in experimental models and in human asthma [43,44]. NF-κB activation occurs in a reply to different stimuli including oxidative stress, inflammatory cytokines, infection, and microbial products. Activated NF-κB translocates from the cytoplasm to the nucleus to upregulate gene expression of proinflammatory cytokines, acute phase proteins, chemokines, and immunoreceptors which are crucial for recruitment of neutrophils and eosinophils [45,46]. Expression of NF-κB was proved by Poynter et al. [44] to be enhanced in bronchiolar epithelial cells that act as a regulator of neutrophil and eosinophil chemokines, macrophage inflammatory protein-2 (MIP-2) and eotaxin. In a model of Dermatophagoides pteronyssinus 2-induced lung inflammation, tiron was found to be effective in suppressing airway inflammation [47].

Results from experimental and human studies found that increased ROS production leads to airway hyper-responsiveness, inflammation,

and tissue injury that can culminate into airway remodeling [48,49]. In the lungs with asthmatic inflammation activation of inflammatory cells like eosinophils and neutrophils is coupled with a buildup of elevated amounts of ROS from these cells with a shift towards higher level of oxidative stress [50]. Mitochondria are also involved in redox homeostasis, ROS and nicotinamide adenine dinucleotide phosphate production, calcium metabolism, and apoptosis [51]. Decreased ATP levels and mitochondrial abnormalities such as damage of cristae and mitochondrial bulging with diminished cytochrome c oxidase action were characterized in the lungs of asthmatic mice [52]. Substances that work as free radical scavengers, as vitamin E dampen these features via abolishing mitochondrial dysfunction [53,54] suggesting that chronic mitochondrial ROS production may be deleterious to mitochondrial function and the airway in experimental asthma [52].

In this study there was an increased oxidative stress in asthmatic mice revealed by elevated concentration of MDA and diminished levels of the antioxidants GSH and SOD than that of control. Tiron and DEXA treatment significantly shifted the balance towards less oxidative stress and higher antioxidant capacity. Wiegman at al. [55] agreed with our investigation and notified that excessive mitochondrial ROS is a contributing factor in abnormalities of airway smooth muscles in patients with chronic obstructive pulmonary disease while targeting mitochondrial ROS with tiron produces a diminution of both the elevated cytokine production and proliferation of airway smooth muscle cells. Mitra and Abraham [56] also reported that the cell permeable superoxide scavenger tiron can inhibit the effects of paraquat, a powerful generator of intracellular superoxide ($O2^{-}$). Tiron was able to block paraquat's enhanced generation of the proinflammatory cytokines TNF-α and MIP-2, enhanced degradation of the inhibitory IκB-α with subsequent increased activation and nuclear transclocation of NF-κB.

Nitric oxide (NO) is a small, ubiquitous molecule with many physiological effects. It is a free radical with an unpaired electron with the ability to react with various molecules including oxygen, superoxide radicals, and transition metals [57]. Superoxide anion ($O2^{-}$) and hydrogen peroxide may limit the bioavailability of NO by the formation of peroxynitrite ($ONOO^{-}$) or as a consequences of uncoupling of endothelial nitric oxide synthase (eNOS) [58]. Reactive nitrogen species (RNS), produced via the reaction of NO with superoxide anions, have powerful pro-inflammatory and oxidizing effects [49].

Inducible nitric oxide synthase (iNOS) is controled at transcriptional level by proinflammatory stimuli. Upregulated iNOS generate huge quantities (nanomolar concentrations) of NO over hours [59]. This upregulation of iNOS is documented in many pathological conditions including lungs of asthmatic, and elevated levels of exhaled NO were found in asthmatics [60]. High levels of NO generated by iNOS is implicated in bronchoconstriction, inflammation and remodeling observed in asthma. On the other hand, NO produced mainly by constitutive isoforms of NOS, has protective effects against bronchoconstriction and remodeling, The dual role of NO in asthma is determined by factors such as the quantity, type of enzyme and location of release [61]. Thus, the impact of NO in asthma is not fully clear with many studies in various experimental models and in humans reporting different results.

Some studies have shown that in asthmatic patients, alveolar macrophages, eosinophils, and polymorphonuclear leucocytes can produce excessive amounts of superoxide and ROS/RNS. Both ROS and RNS may have a vital function in the pathogenesis of asthma and many of pathophysiological changes linked with it [49]. Increased levels of peroxynitrite generated from the interaction of iNOS-derived NO with superoxide lead to increased airway sensitivity and airway inflammation observed in asthma [62]. Asthmatic mice in the existing study presented an elevation in NOx production, an indicator of NO compared with saline-exposed animals. DEXA and tiron reduced these levels indicating lower nitric oxide in both groups. Gabazza et al. [63] illustrated a positive relation among the concentrations of NOx in sputum obtained from patients with asthma and bronchial remodeling. Based on their study, the authors concluded that NO is implicated in progression of airway remodeling. At low levels of L-arginine, iNOS generate NO via its oxygenase moiety and at the same time can synthesize superoxide anions via its reductase moiety resulting in elevated generation of peroxynitrite [64] through the interaction of NO and superoxide. Cao et al. [65] documented a protective effect of peroxynitrite scavenger, Mn TBAP against airway remodeling in murine model that was accompanied by decreasing the apoptosis of epithelial cells. Thus, tiron may have a role in preventing airway remodeling through its action as a superoxide scavenger and by reducing NOx increased in asthmatic mice.

In conclusion, the data of the current research showed no difference between the effect of the mitochondria-directed antioxidant, tiron and that of DEXA in experimental chronic asthma. Based on these findings, the use of tiron could be a reasonable approach to adjuvant therapy of airway remodeling through reversing inflammation and remodeling processes. However, more researches are required to assess this action in the clinical setting.

Conflict of interest

There is no conflict of interest with regard to this research.

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