Open Access Journal

Indian Journal of Medical Research and Pharmaceutical Sciences

September 2015; 2(9) ISSN: ISSN: 2349-5340

Impact Factor (PIF): 2.672

INHALATION OF MYCOBACTERIUM TUBERCULOSIS PROTEIN DERIVATIVE IN A TRIAL FOR TREATMENT OF INDUCED ASTHMA IN GUINEA PIGS. A NOVEL STUDY

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Keywords:
Bronchial asthma; Tuberculin; Ovalbumin; guinea pigs; inhalation.

Abstract

Study objective: Current bronchial asthma therapies have limited long-term effects. Vaccine strategies altering the underlying immune response have been focused upon. Several studies have argued that BCG vaccine may be applied to treatment of allergy by inducing an immune deviation from Th2 to Th1. Based on the same hypothesis; a novel tuberculin inhalation therapy was tried in this study.

Materials and Methods:

30 healthy guinea pigs were randomized into: control group (subjected only to saline inhalation), asthmatic group (Ovalbumin-sensitized and challenged) and tuberculin pretreated group. Respiratory function was monitored and histopathologic examination of the lung tissue was performed using haematoxylin and eosin stain and scanning electron microscopy.

Results: There was a highly significant difference ($p < 0.001$) when comparing the post inhalation airway resistance values (sRAW) and tidal volume (VT), the mean change and mean percentage change in sRAW, and the mean change and mean percentage change in VT, of the sensitized-untreated group with both the control and the sensitized tuberculin pretreated group. Histopathologically: In asthmatic animals there were constriction of small bronchioles and mononuclear cellular infiltration, emphysematous widening of some alveolar sacs. The mean thickness of interalveolar septa was significantly increased; also the mean number of mast cells, eosinophils and macrophages were significantly increased. Using scanning electron microscope numerous goblet cells, accumulated mucous and hypertrophied type II pneumocytes were clearly seen. Pretreatment with tuberculin inhalation minimized most of the previous changes.

Conclusion: tuberculin inhalation can be a promising therapy for asthma that preserves the lung histopathological and functional changes.

Introduction

Bronchial Asthma is an increasing global health problem, it is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. [1] As a consequence of increased production of IL-4, IL-5 and IL-13 by allergen-specific $CD4^+$T helper (Th2 cells) asthma pathology occurs. [2,3]

Current asthma therapies, such as inhaled corticosteroids, β_2-agonists, muscarinic cholinergic receptor antagonists, or anti-leukotrienes, are directed towards symptom relief, reduction or neutralization of effector molecules and

inflammatory mediators. These therapies although symptom reliever effective for acute disease, they have limited long-term effects. [4]

Therefore, an alternative, more effective and long-lasting therapeutic approach for asthma has been focused on, like vaccine strategies, these alter the underlying immune response and convert detrimental allergic responses to protective immune responses, thereby modifying the natural course of the disease.[5]

The BCG (bacillus Calmette–Guerin) vaccine is the most widely used Th1-inducing vaccine.[6] The ability of attenuated TB mycobacteria from which BCG vaccine is derived to modulate the Th2 response has been documented in vitro and in various models of asthma.[7]

Based on these findings, it is hypothesized that inhaled tuberculin (purified protein derivative of mycobacteria) could function similar to BCG; this was tried as a novel immunomodulator therapy.

Materials and methods

Drugs and chemicals:

Tuberculin; a purified protein derivative of a human strain of Mycobacterium tuberculosis (Vacsera, Egypt). Supplied as a transparent solution, each 0.1 ml contains 5 tuberculin units, potassium hydroxyl quinolene sulphate: 10 mcg/0.1 ml and polysorbate (Tween 80): 5 mcg/0.1 ml. Ovalbumin Albumin from chicken egg white, Grade III, aluminium hydroxide powder, pentobarbital Na were purchased from Sigma chemicals, USA.

Experimental animals:

A total of thirty healthy wild type guinea pigs (of an average weight about 450 g) were used. All experimental procedures were approved by the ethical committee of Ain Shams University. Guinea pigs were purchased and the experiments were performed at the pharmacology, histology departments and medical research center, faculty of medicine, Ain shams university.

Study design:

Guinea pigs were randomized into 3 equal groups; control group (9 alive and one died). subjected only to saline injection and inhalation, sensitized-untreated group (experimental model of asthma); only sensitized with intraperitoneal ovalbumin (OVA) injection and 3 weeks later challenged by OVA inhalation, this group included 10 animals (8 alive and 2 died) and sensitized tuberculin- pretreated group, pretreated with tuberculin inhalation 5 i.u. one week before OVA sensitization, this group included 10 animals (8 alive and 2 died).

Sensitization procedure:

An allergen solution containing 100 micrograms OVA and 100 mg $Al(OH)_3$ per ml saline was used. The mixture of allergen solution and Al $(OH)_3$ was gently rotated for 60 min to obtain an alu-gel, and 0.5 ml was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions, and neck. Animals were used experimentally in weeks 3 after sensitization[8].

Tuberculin (PPD) inhalation:

Inhalation of 5 I.U. of tuberculin was done one week before sensitization by OVA and aluminum hydroxide took place.

Experimental procedures:

Three weeks later after injection of OVA, an inhalation challenge with OVA was done and pulmonary functions (sRaw and tidal volume) were measured and finally after two days the animals were sacrificed and the specimens were obtained for histological examination.

Pulmonary functions:

Respiratory function in conscious guinea pigs was monitored using whole-body plethysmography. Changes in airway caliber (bronchoconstriction and bronchodilatation) were recorded as airway resistance and tidal volume. The method

was as described by **Pennock et al.**[9]**.**The basis of the method is the time delay between thoracic and nasal respiration. The increasing phase displacement accompanying the increasing airway resistance was evaluated. Respiratory flow in the body chamber of plethysmograph was measured indirectly by changes in the thoracic gas volume (body box) during respiration. These primary flow signals (nasal and thoracic flow signals) were transmitted via a differential pressure transducer (Validyne D P 45-14) and amplifier in the form of PLUGSYS with CFBA module to a data acquisition / analysis system (Pulmodyn software) from HSE.

Tissue samples:

1. ***Light microscope:*** The specimens were fixed in 10% formol saline for 7 days followed by dehydration in ascending grades of ethyl alcohol, cleared and then, embedded in paraffin. Paraffin section were cut at 4-6µm and were stained by haematoxylin eosin (H & E) [10]
2. ***Scanning electron microscopy:*** lung specimens were collected from the three groups and were rinsed in 2.5% glutaraldhyde fixative solution buffered with 0.1% phosphate buffer for 3 hours. Specimens were then post fixed in osmium tetroxide for 15 minutes. The tissues were then dehydrated through ascending grades of ethanol and the pieces of the lung were dried at critical point dryer using liquid carbon dioxide with Bal-Tec CPD030. The tissues were further mounted on brass studs with aluminum conducting tape and coated with gold in Bal-Tec SCD005. Specimens were examined with Philips XL30 scanning electron microscope operated at 30KV.

Morphometric study:

Was done using image computer analyzing system software known as Optimas, version 6.2. Non-overlapping fields were examined from each animal, the count was done in 2 H&E sections at a magnification of x 1000. The thickness of interalveolar septum was measured in 2 H&E sections from each animal at a magnification power of x 400.

Statistical analysis:

Data were expressed as mean, standard deviation (SD), and 95% confidence interval (95% CI) of the mean. Intergroup differences were compared using one-way analysis of variance (ANOVA) with application of the Tukey honestly significant difference (HSD) test for *post hoc* pairwise comparisons whenever a statistically significant difference was detected. Within-group differences were compared using the paired-samples Student- t test. All P values are two-sided. $P < 0.05$ was considered statistically significant and $P < 0.001$ was considered statistically highly significant.

Results

Mean sRAW before and after inhalation in the three study groups:

Control guinea pigs subjected only to saline inhalation, showed no significant difference between baseline mean sRAW (2.93 $cmH_2O.s$) and post-inhalation mean sRAW (2.72 $cmH_2O.s$) with 95% CI (2.6 to 3.26) and (2.46 to 2.98) for both respectively. In the sensitized untreated guinea pigs; ovalbumin inhalation increased baseline mean sRAW from 3.09 to mean post-inhalation sRAW of 16.94 cmH2O (P<0.001) 95%CI for both means was 2.72 to 3.45, and 15.11 to 18.76 respectively. Among sensitized tuberculin pretreated animals baseline mean sRAW increased from 3.11 cmH2O.s (95%CI: 2.87 to 3.35) to 8.69 cmH2O.s after ovalbumin inhalation, (95%CI: 7.44 to 9.94) still with statistically highly significant difference (P<0.001). The post-inhalation sRAW was significantly higher in the sensitized untreated group compared with both the control group (P<0.001) and the sensitized pretreated group (P<0.001). (Table 1)

Mean V_T before and after inhalation in the three study groups:

In control animals no significant difference was found between baseline (2.64 ml) and post-inhalation (2.80ml) mean V_T with 95% CI (2.37 to 2.92) and (2.61 to 2.99) for both respectively. Statistically significant difference (P value = 0.002) was detected among sensitized untreated animals between baseline mean V_T; 2.73 ml (95% CI: 2.28 to 3.17) and ovalbumin post-inhalation mean V_T; 1.48 ml (95% CI: 1.12 to 1.83). In the sensitized tuberculin pretreated guinea pigs; ovalbumin inhalation decreased baseline mean V_T from 2.89 ml to mean post-inhalation V_T of 2.54ml, with no statistically significant difference, 95%CI for both means was 2.52 to 3.26, and 2.24 to 2.83 respectively. (Table 1)

Mean percentage change in sRAW (% Δ sRAW) among the three studied groups:
The mean percentage change in sRAW for control animals was -4.97% , for sensitized-untreated animals was 453.09% and for sensitized tuberculin pre-treated group was 179.7% with 95%CI -20.80 to 10.86, 394.77 to 511.40 and 142.92 to 216.50 for each respectively. Comparison between the three studied groups showed statistically highly significant difference (p <0.001) (Table 1).

Mean percentage change in tidal volume (% Δ V_T) among the three studied groups:
The mean percentage change in V_T for control animals was 6.81%, for sensitized-untreated animals was -42.69% and for sensitized tuberculin pretreated group was -10.54% with 95% CI -1.03 to 14.66, -63.89 to -21.48 and -24.20 to 3.13 for each respectively. Comparison between the three studied groups showed statistically highly significant difference (p <0.001) (table 1).

Table 1. In-vivo measures: Inter-group comparisons

	Control Group (n=9)			Sensitized-Untreated Group (n=8)			Tuberculin Pretreated Group (n=8)			
Variable	Mean	SD	95% CI	Mean	SD	95% CI	Mean	SD	95% CI	P value §
Baseline sRAW ($cmH_2O.s$)	2.93	0.43	2.6 to 3.26	3.09	0.44	2.72 - 3.45	3.11	0.29	2.87 to 3.35	0.598
Postinhalation sRAW ($cmH_2O.s$)	2.72	0.34	2.46 to 2.98	16.94*†	2.18	15.11 - 18.76	8.69*	1.50	7.44 to 9.94	<0.001
Δ sRAW($cmH_2O.s$)	-0.21	0.59	-0.67 to 0.24	13.85*†	1.98	12.20 to 15.50	5.58*	1.42	4.39 to 6.77	<0.001
Δ sRAW (%)	-4.97	20.59	-20.8to10.86	453.09*†	69.75	394.77 to 511.4	179.7*	44.00	142.92 to 216.50	<0.001
Baseline V_T (ml)	2.64	0.35	2.37 to 2.92	2.73	0.54	2.28 to 3.17	2.89	0.45	2.52 to 3.26	0.537
V_T post-inhalation (ml)	2.80	0.24	2.61 to 2.99	1.48*†	0.43	1.12 to 1.83	2.54*	0.35	2.24 to 2.83	<0.001
Δ V_T (ml)	0.16	0.24	-0.03 to 0.34	-1.25*‡	0.76	-1.88 to -0.62	-0.35	0.55	-0.81 to 0.11	<0.001
Δ V_T (%)	6.81	10.20	-1.03to 14.66	-42.69*‡	25.36	-63.89 to -21.48	-10.54	16.35	-24.20 to 3.13	<0.001

§Estimated with one-way ANOVA.

*P<0.001 versus Control Group (Tukey's HSD test).

†P<0.001 versus Sensitized-Treated Group (Tukey's HSD test).

‡P=0.009 versus Sensitized-Treated Group (Tukey's HSD test).

Indian Journal of Medical Research and Pharmaceutical Sciences
September 2015; 2(9) ISSN: ISSN: 2349-5340
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Histopathological results:
In asthmatic guinea pigs there were cellular debris in the lumina of some bronchi and bronchioles, infiltration by mononuclear cells, constricted small bronchioles and thick interalveolar septa (Fig. 1A and B), some alveoli and alveolar sacs showed emphysematous dilatation (Fig. 1-C). Scanning electron microscope showed thick interalveolar septa and hypertrophied type II pneumocytes (Fig. 1-D).

Treated guinea pigs with tuberculin: the lung tissue specimens obtained from this group showed that most of the interalveolar septa were seen to be thin. Few interalveolar septa were thickened and showed mononuclear cell infiltration (Fig. 1-E and F)

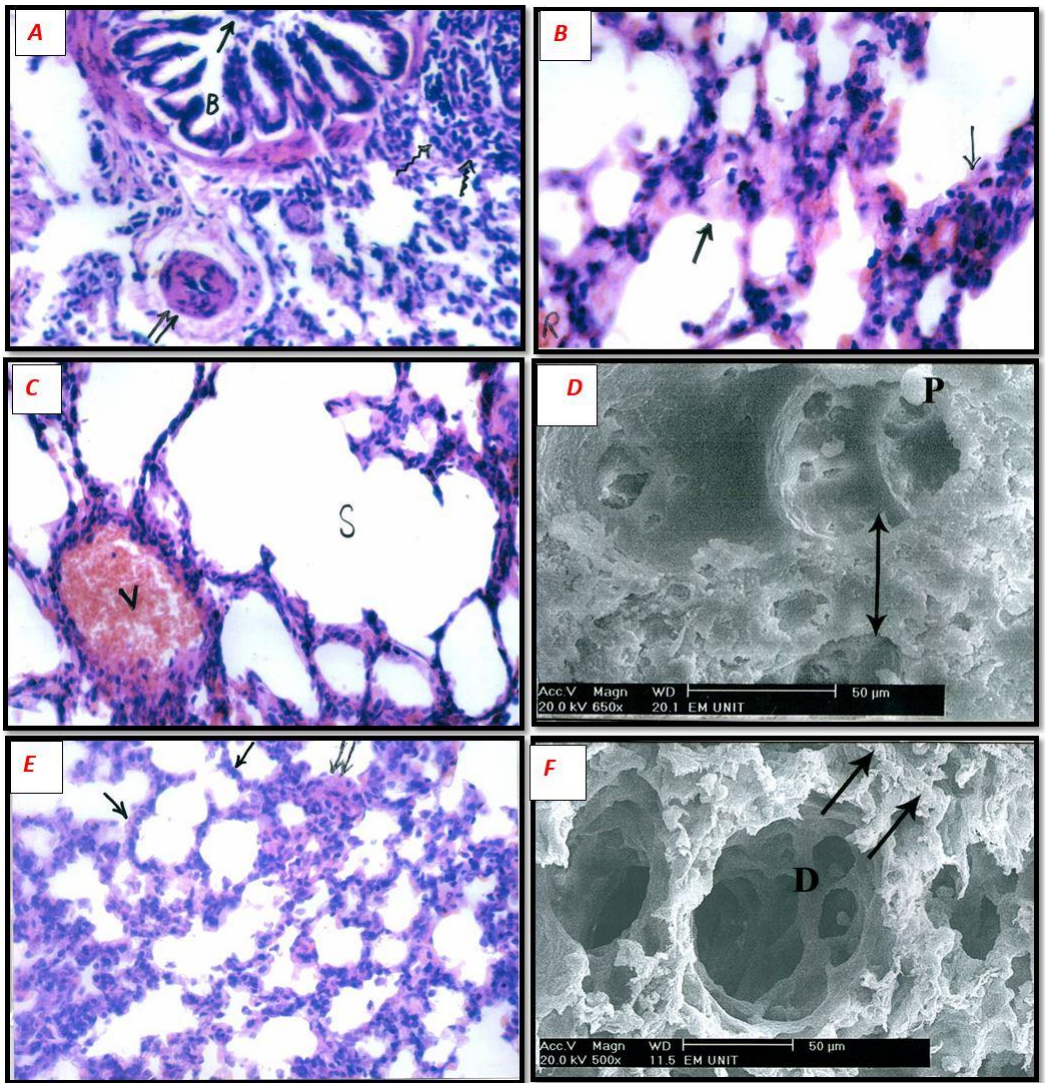

Fig 1 A- showing cellular debris in bronchial (B) lumen (↑), mononuclear cellular infiltration (~~~>) and a constricted bronchiole (↑↑). B- Thickened intra-alveolar septa (↑). C- Vascular congestion (v) and emphysematous dilatation of alveolar sac (s). D- Scanning electron micrograph showing thick intraalveolar septa (↕) and hypertrophied type II pneumocyte. E- Some areas showing thin interalveolar septa (↑) other areas shows thick interalveolar septa (↑↑). F- Alveolar duct (D) and thin interalveolar septa (↑)

Intergroup comparison of microscopic findings revealed a highly significant statistical difference as regards Eosinophil count, mast cell count and interalveolar septum thickness between sensitized untreated group in comparison to both control group (p< 0.001) and sensitized tuberculin pretreated group (p< 0.001) (Table 2)

Table 2. Intergroup comparison of microscopic measures

	Control Group (n=9)			Sensitized-Untreated Group (n=8)			Tuberculin pretreated Group (n=8)			
Variable	Mean	SD	95% CI	Mean	SD	95% CI	Mean	SD	95% CI	P value§
Eosinophil Count (cells/HPF)	1.17	0.66	0.66 to 1.68	7.56*†	1.35	6.44 to 8.69	3.19‡	0.70	2.60 to 3.78	<0.001
Mast cell count (cells/HPF)	1.50	0.71	0.96 to2.04	8.00*†	2.24	6.13 to 9.87	4.06#	1.18	3.08 to 5.05	<0.001
Thickness of Interalveolar septum (mm)	4.50	0.75	3.92 to 5.08	130.01*†	30.16	104.79 to 155.22	37.88■	7.49	31.62 to 44.14	<0.001

§Estimated with one-way ANOVA.
*P<0.001 versus Control Group (Tukey's HSD test).
†P<0.001 versus Sensitized tuberculin pretreated Group (Tukey's HSD test).
‡P=0.005 versus Control Group (Tukey's HSD test).
P=0.001 versus Control Group (Tukey's HSD test).
■P=0.002 versus Control Group (Tukey's HSD test).

Discussion

Asthma prevalence has increased over the past decades. The increase in allergic diseases especially in the developed countries could be explained by the "hygiene hypothesis" where there are cleaner environment and fewer childhood infections. [11-14] Shirakawa et al, reported an inverse association between tuberculin responses and atopic disorders in Japanese children. [15] Since then attention has been directed towards mycobacterial exposure and asthma relationship. Positive data for the capacity of various preparations of mycobacterial antigens to prevent allergic sensitization have been shown consistently in some studies with mouse model of asthma. [16-21]

Hattori et al [22], investigation results indicated that the intranasal application of PPD, and the subsequent induction of IFN-γ, inhibits the initiation, but not the exacerbation, of allergic rhinitis in mice. This suggested that pathogen-derived antigens (PPD) and not only BCG have potential for use in the prevention and prophylaxis of allergic rhinitis.

We designed the present study to more closely investigate the beneficial functional and histopathological effects of PPD on OVA-sensitized guinea pig model of asthma and to the best of our knowledge this is the first trial.

Our results show convincing evidence that bronchoconstriction in response to nebulised OVA in sensitized treated guinea-pigs was reduced by tuberculin inhalation. There was a highly significant difference when Comparing the post-inhalation sRAW, the Mean change in sRaw and the Mean percentage change in sRAW of the sensitized-untreated group with both the control group (P<0.001) and the sensitized pretreated group (P<0.001).

In the sensitized tuberculin pretreated guinea pigs; mean tidal volume was slightly decreased after ovalbumin challenge when compared to untreated animals. There was a highly significant difference when comparing the post-inhalation V_T, the Mean change in V_T and the Mean percentage change in V_T of the sensitized-untreated group with both the control group (P<0.001) and the sensitized-treated group (P<0.001).
Our data are consistent with the notion that BCG vaccination induces Th1 responses, which subsequently inhibits allergen-specific Th2 responses, leading to attenuation of allergic airway inflammation, and protective effect in pulmonary functions.

Su et al. also demonstrated that during the nebulised OVA challenges, the tidal volume of the OVA+BCG and BCG groups were higher, and the airways hyper-reactivity appeared to be much better than those of the control and OVA groups. [23]

We reported findings similar to those previously shown by Shen et al, where neonatal vaccination with BCG inhibited allergen-induced airway hyperreactivity (AHR) in both early- and late-challenged mice. Penh (Enhanced Pause) values assessed by whole-body plethysmography, in the mice which received BCG vaccination were paralleled with that of saline controls and were significantly lower than that of OVA group of mice in response to 25 or 50 mg/ml MCh ($P < 0.05$).[24]

Consistent with this; Lagranderie et al [25,] demonstrated that EFD (extended freeze dried) BCG treatment reduces dynamic compliance and resistance significantly ($P < 0.001$) in mechanically ventilated chronic model of asthma. OVA-sensitized mice were EFD BCG treated after 3 OVA challenges. Twenty- four hours after the last OVA-challenges Penh values were recorded after increasing doses of metacholine.

Another study[26] performed in a mouse model of allergic airway inflammation, administrating mycobacterial treatment (both Bacillus Calmette-Guérin and M. vaccae) substantially attenuates airway resistance associated with LAR (late allergic response) without affecting EAR (early allergic response), this could be explained by the different species, methods of sensitization and the different doses of M.vaccae used.

Histological examination of lung specimens using L.M. and scanning electronmicroscope showed that in the asthmatic animals some of the bronchi showed hypertrophy of the lining epithelium. Scanning EM showed large areas with irregular, disrupted cilia. Barns[27] explained that this epithelial damage and shedding could contribute to airway hyper-responsiveness in several ways: these include loss of barrier function, which may allow penetration of allergens; loss of enzymes that break down inflammatory mediators; and exposure of sensory nerves, which may lead to reflex neural effects on the airway.

Regarding goblet cells, the control group scanning EM showed few ones in between the lining epithelium. On the other hand, in the asthmatic group, goblet cells were seen to be numerous in other areas with clearly seen accumulated mucous. The tuberculin treated group specimens showed few goblet cells compared with the untreated one. Zhang et al., [28] reported similar effect of reduced goblet cells hyperplasia of in the murine groups treated with nebulized inhalation of inactivated Mycobacterium phlei.

This agreed with Ozdemir et al., [29], whom results analysis showed that the asthmatic group goblet cells number was significantly higher than that in the immunized group (immunized with multiple subcutaneous injections of heat killed Mycobacterium vaccae), but it was also found that those of the immunized group were significantly higher than that in the control group, this disagrees with the present study. The difference could be attributed to the different methods of sensitization and challenge.

Using light microscope, lung tissue of asthmatic animals showed few patent alveoli. Cellular debris were demonstrated in the lumina of bronchi and bronchioles. Most of small bronchioles were seen to be constricted. Scanning electronmicroscope showed protruded Type II pneuomocytes into the alveolar lumina due to hypertrophy of these cells.Thickened interalveolar septa were clearly seen in the asthmatic group. Thickened septa showed infiltration by mononuclear cells, congested blood vessels and edema fluid.

In the tuberculin pretreated group, cellular infiltration was seen in few areas. This agreed with Zhang et al., [28]who reported that lung tissues examination by H. &E of the asthma model showed a significant infiltration of total inflammatory cells around airways and blood vessels and that the majority of the infiltrated inflammatory cells were eosinophils. On the other hand, the administration of inactivated Mycobacterium phlei reduced the infiltration of inflammatory cells in the peribronchial and perivascular areas compared with the asthma model mice. Hopfenspirger and coworkers[26] also noticed marked edema and inflammatory infiltration in H&E-stained lung sections from OVA-

sensitized control animals while Lungs of the BCG-and M. vaccae-treated mice showed an absence of inflammation and only mild edema.

Regarding tuberculin group, there was a significant decrease of both eosinophil and mast cell mean counts compared with the asthmatic group. This agreed with Koh el al., [30] who found that Eosinophil count in bronchoalveolar lavage fluid was significantly decreased in rats infected with BCG compared with the uninfected asthmatic untreated rats ($P<0.01$).

Statistical analysis showed that the thickness of interalveolar septa mean value had increased significantly in the asthmatic group compared to the control one, while it decreased significantly in the treated group compared to the asthmatic one. This matched with Ozdemir et al., [29] results who found decreased thickness of the basement membrane comparing the immunized group with the asthmatic one.

Sue et al.,[23] noticed that the alveolar cells thickened, especially in the OVA and control groups. The structure of the alveoli was damaged, including an accumulation of inflammatory cells and secretory fibrin. The group of guinea pigs immunized with BCG also had obstruction of the alveoli. Inflammatory cells accumulated as nodules, surrounding the bronchi, and caused progressive emphysema. The OVA+BCG group showed little hyperplasia on the bronchial epithelia, with no loss of alveolar structure.

Conclusion

Our data support the view that mycobacterial infections have the potential to suppress inflammatory allergic responses in animal model of asthma. We discovered that tuberculin (PPD) can cause bronchodilatation following a bronchoconstrictor stimulus which may explain its beneficial clinical and histopathological effects making it a promising antiasthma alternative treatment.

Acknowledgements

No funds were granted for this work and authors have no conflict of interest.

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