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Preclinical evaluation of co administered bronchodilators and corticosteroid for the treatment of asthma

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ABSTRACT

Asthma is chronic inflammatory disorder of the airways characterized by acute exacerbation of coughing, dyspnoea, wheezing and chest tightness particularly at night as well as in the early morning .Asthma is also widely recognized as a disease of lung characterized by reversible bronchoconstriction, elevated basal airway tone and lymphocyte (Eosinophilis) activation and accumulation, epithelial cell dysfunction and damage, smooth muscle and submucosal gland hypertrophy, submucosal fibrosis, airway wall edema, mucus overproduction and episodes of non-specific airway hyper-responsiveness to spasmogens . For managing asthma attacks, symptomatic relief is foremost requirement. Bronchodilators and corticosteroids are used simultaneously for the effective treatment of acute and chronic asthma. In the present study, effect of combination of salbutamol (bronchodilator) and Prednisolone (corticosteroid) in different ratios (100:00, 70:30, 50:50, 30:70 and 00:100) was studied pre clinically to predict suitable ratio of both the drugs for the effective treatment of asthma. For evaluation, animal models used were Passive paw anaphylaxis in rats, Milk- induced eosinophilia in mice, isolated goat trachea, Broncho alveolar levage and Lung histopathology in mice. From this pre clinical studies it was found that, combination of Salbutamol (bronchodilator) and Prednisolone (corticosteroid) was effective for the treatment of acute and chronic asthma in all ratios but, at the same time the most effective ratio of drugs Salbutamol: Prednisolon was found to be70:30 which suggest that, corticosteroids at lower concentration are also equally effective so unnecessary use of high doses must be avoided. And as asthmatic attacks at early morning hrs are result of changes in circadian rhythm, this study warrants us to prepare chronopharmaceutical drug delivery system for the same combination of drugs and evaluate.

Keywords: Asthma, bronchodilator, corticosteroid, Salbutamol, Prednisolone and chrono pharmaceuticals

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INTRODUCTION

Asthma is chronic inflammatory disorder of the airways characterized by acute exacerbation of coughing, dyspnoea, wheezing and chest tightness particularly at night as well as in the early morning [1] .Asthma is also widely recognized as a disease of lung characterized by reversible bronchoconstriction, elevated basal airway tone and lymphocyte (Eosinophils) activation and accumulation, epithelial cell dysfunction and damage, smooth muscle and submucosal gland hypertrophy, submucosal fibrosis, airway wall edema, mucus overproduction and episodes of non-specific airway hyper-responsiveness to spasmogens [2] . In the United States alone, asthma affects almost 17 million people, and this is a 75% increase in the last 20 years. This means that about 1 out of every 20 adults and close to 1 out of 13 children today have asthma. An alarming fact is that since 1980, asthma in children under age 5 has risen remarkably. In school age children, asthma has risen by 75%. India alone has an estimated 15-20 million asthmatics. Mortality data from developed countries show that the rate varies from 0.1- 0.8 per 100,000 persons aged 5-34. [3] The worldwide prevalence of allergic diseases has increased during the last two decades [4, 5]. Acute allergic reactions result from the release of preformed granule-associated histamine and tryptase, membrane-derived lipid mediators (leukotrienes, prostaglandins and platelet activating factor), cytokines and chemokines; release of these signals occurs when an allergen interacts with IgE bound to the membranes of mast cells and basophils, which leads to events such as smooth muscle contraction, vasodilatation, increased vascular permeability and mucous hypersecretion [6]. Thus, when allergic disease occurs, affected tissue is infiltrated by cells with a Th2-type cytokine profile (such as IL-4, IL-5, IL-9 and IL-13 [7,8], which favors the synthesis of IgE) and mast cells, lymphocytes and eosinophils are activated, all of which leads to inflammation and disease [9,11].

Salbutamol which is β2 agonist used in acute asthma for Symptomatic relief during maintenance therapy of asthma and other conditions with reversible or irreversible airways obstruction (including COPD and bronchitis) [10]. Prednisolone is a corticosteroid drug with predominantly glucocorticoid and low mineralocorticoid activity, making it useful for the treatment of a wide range of inflammatory and auto-immune conditions such as asthma, uveitis, Pyoderma Gangrenosum, rheumatoid arthritis, ulcerative colitis[11]. For managing asthma attacks, symptomatic relief is foremost requirement. Bronchodilators and corticosteroids are used simultaneously for the effective treatment of acute and chronic asthma [12].

In the present study, effect of combination of salbutamol (bronchodilator) and Prednisolone (corticosteroid) in different ratio was studied pre clinically to predict suitable ratio of both the drugs for the effective treatment of asthma. For evaluation, animal models used were Passive paw anaphylaxis in rats, Milk- induced eosinophilia in mice, isolated goat trachea, Broncho alveolar levage and Lung histopathology in mice.

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MATERIALS AND METHOD

Drugs

Prednisolone (Wyseolon, Wyeth, India), Salbutamol (Asthalin, Cipla Ltd.,Mumbai)

Animals

Wistar rat (200-250 g) of either sex were used for present study. Animal were kept under a 12 h light/ 12 dark cycle, with free food and water ad libidum. Male albino mice (Swiss strain) weighing 22- 25 g were housed under standard laboratory condition in a group of five each. Animals had free access to food and water. The Institutional Animal Ethical committee (IAEC) has approved the protocol of the study.

Chemicals

Aluminium hydroxide gel, Dexamethasone, Salbutamol, Prednisolone, egg albumin, etc.

Selection of doses

Salbutamol and prednisolone in the human doses of 8 and 30 mg were chosen and different ratio of these drugs [50: 50 (T1), 70:30 (T5) and 30:70 (T2)] along with 100% doses of each drug T3 and T4 respectively were employed for the further study.

EVALUATION OF ANTIASTHMATIC ACTIVITY

In-vitro experimentation

Effect of combination of salbutamol and prednisolone on isolated goat tracheal chain preparation

Isolated adult goat tracheal tissue was obtained immediately after slaughterhouse of the animals. Trachea was cut into individual rings and tied together in series to form a chain. Trachea was suspended in bath of Krebs solution and was continuously aerator at 37 + 0.5^{0}C. DRC of histamine was recorded with different concentrations. DRC of histamine was repeated in presence of different concentrations of salbutamol and prednisolone. [8]

In-vivo experimentation

Milk- induced leukocytosis in mice

Albino mice were divided into seven groups (n=5). Group I served as negative control receives milk induced stress (boiled and cooled, 4 ml/kg, s.c.). Group II, III, IV, V, VI received T1, T2, T3, T4, T5 doses respectively. Blood samples were collected from retro orbital plexus under

light ether anesthesia, the eosinophil count is done in each group before drug administration and 24 h after the milk injection (boiled and cooled, 4 ml/kg, s.c.). Difference in the eosinophil count before and 24 h after milk administration was noted using modified method described by Brekhman[11].

Histamine induced bronchoconstriction in guinea pig

Wistar rats were divided into seven groups (n=5). Group I served as negative control receives milk induced stress (boiled and cooled, 4 ml/kg, s.c.). Group II, III, IV, V, VI received T1, T2, T3, T4, T5 doses respectively. The guinea pigs fasted for 24 h were exposed to an atomised fine mist of 2% histamine dihydrochloride aerosol (dissolved in normal saline) using nebulizer at a pressure of 300 mm Hg in the histamine chamber (24 x 14 x 24 cm, made of perplex glass). Guinea pigs exposed to histamine aerosol showed progressive signs of difficulty in breathing leading to convulsions, asphyxia and death. The time until signs of convulsion appeared is called pre-convulsion time (PCD). By observation experience was gained so that the preconvulsion time can be judged accurately. As soon as PCD commenced, animals were removed from the chamber and placed in fresh air to recover.

Passive paw anaphylaxis in rats

Wistar rats of either sex were administered with three doses of 100 µg of egg albumin (s. c.) adsorbed on 12 mg of aluminum hydroxide gel prepared in 0.5 ml of saline on 1st, 3rd, 5th day. On 10th day of sensitization blood was collected from the retro orbital plexus and collected blood was allowed to clot and the serum was separated by centrifugation at 1500 rpm. Animals were divided into five groups (n = 5). Animals belonging to group I served as control and were administered only the vehicle (10ml/kg p. o.). Animals belonging to groups II, III, IV, V, VI received drug combination T1, T2, T3, T4, T5. Animals of group VII, as positive control group received dexamethasone (0.27mg/kg p. o.). The animals were passively sensitized with 0.1ml of the undiluted serum into the left hind paw of animals. The contra lateral paw received unequal volume of saline. Drug treatment was given 24 hr after sensitization. Animals were challenged in the left hind paw with 10µg of egg albumin in 0.1ml of saline, and the paw inflammation was measured using a plethysmometer. The difference in the reading prior to, and after antigen challenge represented the edema volume and the percent inhibition of volume was calculated by using the following formula. [8]

$$\text{Percent Inhibition} = 1 - (Vt / Vc) \times 100$$

Vt = Mean relative change in paw volume in test group
Vc = Mean relative change in paw volume in control group.

Prior drug treatment animals were sensitizes with serum. Next 24 hours, after drug treatment animals again challenged for 10 µg egg albumin and edema inhibition was calculated. [8-10]

Broncho-alveolar levage and lung histology in mice

Albino mice of either sex were divided into seven groups containing five animals each (n=5).All animals were sensitized by an intraperitonial injection of 1ml alum precipitate antigen containing 20µg of ova albumin and 8mg of alum suspended in 0.9% of sodium chloride solution. A booster injection of this alum-albumin mixture was administered 7 days later. Non sensitized animal were injected with alum only (Group I). Seven days after (15 days) the second injection, animal was exposed to aerosolized oval albumin (1%) for 30 min. Animals belonging to groups II, III, IV, V, VI received drug combination T1, T2, T3, T4, T5. Animals of group VII, as positive control group received dexamethasone (0.27mg/kg p.o.) 5 hr before antigen challenge. The mice were sacrificed at the end of study (24hr after sensitization) and trachea catheter was inserted in trachea. Brochoalveolar lavage fluid (BALF) was collected by lavaging the lung with two aliquots 5ml of 0.9% of sodium chloride solution. Total recovery volume per mice was approximately 5ml. Total leukocyte eosinophils and neutrophils were counted under microscope and histopathologic evaluation of lung tissue was carried out. [12]

Statistical analysis

The statistical analysis was performed by using one-way analysis-of-variance (ANOVA) followed by Dunnett's test for individual comparison of groups with control.

RESULTS AND DISCUSSION

In the present study, effect of combination of Salbutamol (bronchodilator) and Prednisolone (corticosteroid) in different ratios (100:00, 70:30, 50:50, 30:70 and 00:100) was studied pre clinically to predict suitable ratio of both the drugs for the effective treatment of asthma. For evaluation, animal models used were invitro model isolated goat trachea, Passive paw anaphylaxis in rats, Milk- induced eosinophilia in mice, Bronchoalveolar lavage and Lung histopathology in mice.

Prednisolone is a corticosteroid drug with predominantly glucocorticoids and low mineralocorticoid activity, making it useful for the treatment of a wide range of inflammatory and auto-immune conditions such as asthma, uveitis, Pyoderma Gangrenosum, rheumatoid arthritis, ulcerative colitis. Salbutamol is β_2 agonist used in acute asthma symptom relief during maintenance therapy of asthma and other conditions with reversible or irreversible airways obstruction (including COPD and bronchitis). [13]

The dose dependent contraction by spasmogens such as histamine (0.1-102.4 mg), acetylcholine (0.1-12.8 µg), and barium chloride (0.1-51.2 mcg) using goat tracheal chain preparation.[14] Histamine antagonism modulated by the relaxing factors involved and may be due to the suppression of histamine H1-receptor. The present work aimed at justifying and investigating the potential of salbutamol and prednisolone in antagonizing the histamine induced contractions which have shown a significant relaxation of goat trachea indicated by right shift of DRC of histamine. Thus it can be concluded from the effect of salbutamol and

prednisolone on isolated goat tracheal chain preparation that both drugs possess antihistaminic (H1-receptor antagonist) action. [15]

In the present study the DRC of histamine (10 µg / ml) was plotted % response vs concentration. DRC was shifted to right by salbutamol and prednisolone at the concentration 10 µg/ ml. The antihistaminic activity offered by prednisolone is greater than that of salbutamol.

After parental administration of milk there is abnormal increase in total leukocyte count (TLC) which is termed as leukocytosis. The drug which is able to produce significant protection against raised levels of TLC can disaggregates the stressful condition and can be termed as the antistress agent producing the adaptogenic activity in vivo. From the present investigation it can be concluded that there is significant reduction in the elevated TLC due to treatment with prednisolone and combination of it with salbutamol. The adaptogenic activity was found to be in the order of T2> T1>T4> T5> T3 which reflected the role of prednisolone as adaptogenic agent and the combination of salbutamol: prednisolene in the ratio of 70: 30 is producing better activity than that of 30: 70.

The characteristic features of bronchial asthma include airway hyperresponsiveness (AHR) to nonspecific stimuli, bronchoalveolar lavage (BAL). eosinophilia, and the predominance of a type 2 cytokine profile. In about 40% of asthmatics, the late allergic response (LAR) .has also been reported. [16-18]

Prednisolone treated group showed maximum protection against emphysematic condition and infilteration of lymphocytes, monocytes and macrophages. There was pronounced emphysema and denudation of epithelium, necrosis of epithelium in salbutamol treated group. In combination treated groups the salbutamol: prednisolone 30: 70 produced better protection against the necrosis and infilteration when compared to 70: 30 and 50:50 treated group respectively in order of T3> T2> T5> T1> T4.

Asthma is characterized as a disease involving airway smooth muscle spasm and inflammation. Eosinophils can be found in the airways of asthmatics, and are known to release several mediators of inflammation including eosinophilic cationic protein (ECP) and major basic protein (MBP).

Following challenge to OVA, mice that were previously sensitized, but untreated demonstrated significantly more reactive airways.

In the present study, BAL fluid collected from the sensitized control mice contained greatly increased numbers of eosinophils as compared to all other groups of mice.

In the present study, treatment with salbutamol and prednisolone demonstrated significant reduction in number of inflammatory mediators like macrophages and basophils when compared to the inducer group. Treatment with the combination produced more

decrease in the inflammatory cell count in BALF. The histo pathological studies substantiate the finding that combination treatment produces beneficial effects as anti allergic and antianaphylactic as there is reduction in the emphysematous spaces and recruitment of the inflammatory mediators like eosinophils, monocytes and other leukocytes. [19, 20]

To our knowledge, this is the first report of the effect of drug combination of salbutamol and prednisolone on recruitment of the inflammatory mediators involved in asthma.

Allergic asthma is a chronic inflammatory process occurring due to exposure of allergen resulting in the activation of T-lymphocyte with subsequent release of inflammatory mediators. Immuno-modulating agents are useful in the treatment of asthma by inhibiting the antigen-antibody (AG-AB) reaction and there by inhibiting the release of inflammatory mediators. In case of passive paw anaphylaxis, there is immunological stimulation by ova albumin and the antibodies raised against the antigen are injected locally into the paw of rat. Local antigen antibody reaction in the rat paw manifested into the inflammation and paw edema. In the present investigation, as the treatment with the prednisolone and combination had demonstrated the inhibition in the paw edema, it can be concluded that the combination of salbutamol and prednisolone 70: 30 produced better % inhibition in paw volume. The probable mechanism of action could be the optimum antianaphylactic and antiallergic as well as anti-inflammatory activity offered by combination treatment.

In the present study, histamine aerosol significantly produced bronchoconstricition in guinea pigs which was evident by reduced preconvulsive dyspnea time (PCT). The treatment with salbutamol and combination of salbutamol with prednisolone in 70:30 and 50: 50 produced significant prolongation of PCT thereby produced the protection against hypoxia by bronchodialtion. The activity demonstrated by salbutamol : prednisolone 70: 30 was highest followed by salbutamol alone treated group and group treated with 50: 50 respectively. It can be concluded that there is synergistic bronchodilator activity with 70: 30 treatment.

CONCLUSION

From this pre clinical studies it was found that, combination of bronchodilator with corticosteroid was effective for the treatment of acute and chronic asthma in all ratios. But, at the same time the most effective ratio of drugs Salbutamol: Prednisolone was found to be70:30 which suggest that, corticosteroids at lower concentration are also equally effective and unnecessary use of high doses must be avoided as asthmatic attacks at early morning hours are result of changes in circadian rhythm and at morning hours steroidal level in the blood is also high because of changes in circadian rhythm and use of high doses may be harmful. So this study warrants us to prepare chronopharmaceutical drug delivery system for the same combination of drugs and evaluate.

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Figure 1: Effect of combination of salbutamol and prednisolone on Isolated Goat Tracheal Chain

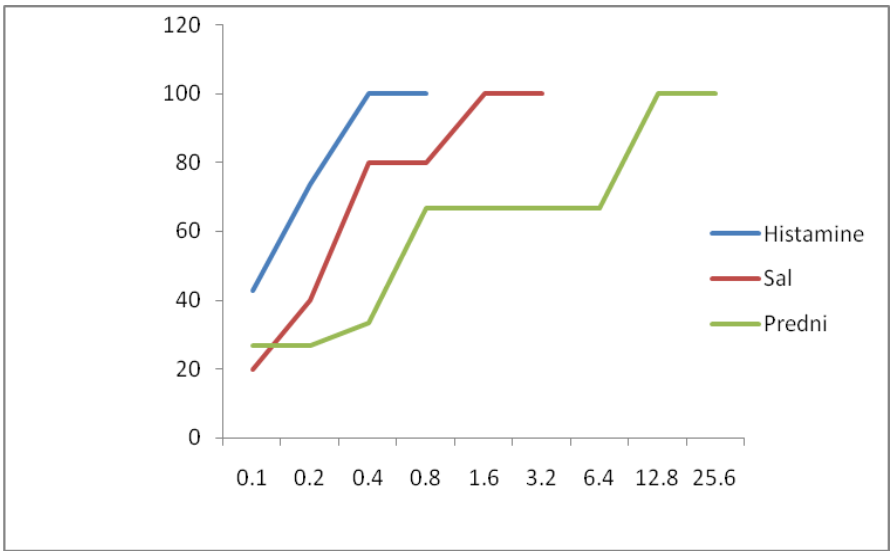

Table 1: Milk- induced leukocytosis

Groups	Treatment	Total leukocyte count
I	Inducer Control	14933.00 ±2021.8
II	T1	5766.70 ±1967.5*
III	T2	5603.30 ±2233.80*
IV	T3	13267.00 ±2445.00
V	T4	5833.30 ±876.23*
VI	T5	11433.00 ±1317.00

Table 2: Histamine induced broncho constriction in guinea pigs.

S. N.	Treatment	PCD (Sec)
I	Inducer Control	77.2 ± 1.93
II	T1	154. 4 ±4.65**
III	T2	135.8 ±2.70**
IV	T3	163.4 ±7.33**
V	T4	126.8 ±4.64**
VI	T5	178.8± 4.64**

n = 5; *p<0.05, **p<0.01, compared with control group (ANOVA followed by Dunnett's test)

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Table 3: Effect of on passive paw anaphylaxis in rat

Groups	Treatment	Difference in paw volume (ml)			
		1 hr	2 hr	3 hr	4 hr
I	Control	0.63 ±0.05	0.69 ± 0.02	0.72 ± 0.05	0.77 ± 0.04
II	T1	0.13 ±0.06**	0.09 ±0.03**	0.05 ±0.01**	0.01 ±0.05**
III	T2	0.29 ±0.07**	0.11 ±0.05**	0.09 ±0.02**	0.03 ±0.01**
IV	T3	0.61 ±0.04	0.26 ±0.05**	0.08 ±0.05**	0.02 ±0.01**
V	T4	0.33 ±0.02**	0.26 ±0.01**	0.08 ±0.05**	0.02 ±0.01**
VI	T5	0.27 ±0.05**	0.31 ±0.04**	0.21 ±0.08**	0.09 ±0.05**
VII	Dexamethasone	0.20 ±0.05**	0.34 ±0.11**	0.38 ±0.05*	0.14 ±0.02**

n = 5; *p<0.05, **p<0.01, compared with control group (ANOVA followed by Dunnett's test)

Table 4: Broncho alveolar levage and lung histology in mice

	Group	Total count / microliter	PMN	LYM	Mon
I	Control	6058 ±12.069	55.200 ±0.8602	60.200 ±0.7348	9.200 ±0.3742
II	T1	6490 ±118.36**	41.000 ±0.7071**	50.000 ±0.7071**	3.000 ±0.4472**
III	T2	4060 ±57.879**	49.000 ±1.000**	43.800 ±1.241**	2.400 ± .2449**
IV	T3	3530 ±25.495**	42.400 ±0.9274**	49.600 ±2.205**	2.600 ±0.2449**
V	T4	5520 ±46.63**	38.000 ±1.140**	49.800 ±1.393**	3.200 ±0.3742**
VI	T5	3520 ±46.368**	45.000 ± 1.000**	48.600 ±1.166**	2.600 ±0.2449**
VII	Dexamethasone	3843 ±18.682**	40.200 ±0.7348**	40.000 ±0.7071**	2.400 ±0.2449**

n = 5; *p<0.05, **p<0.01, compared with control group (ANOVA followed by Dunnett's test)

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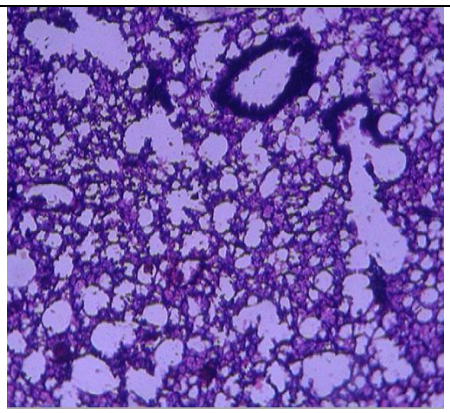
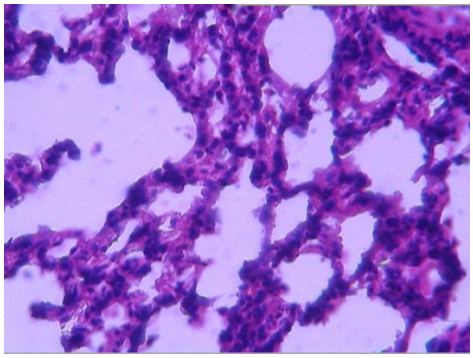
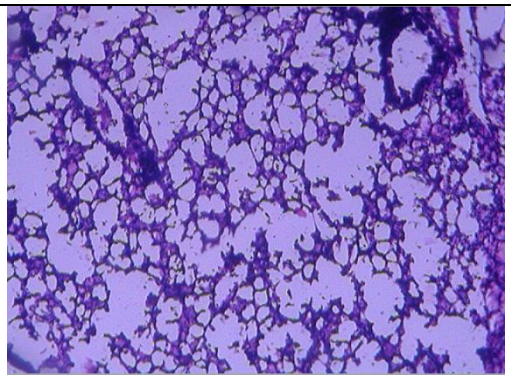
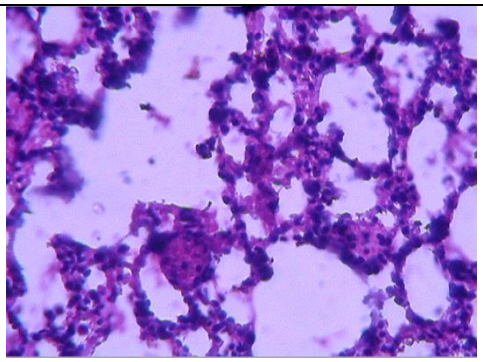
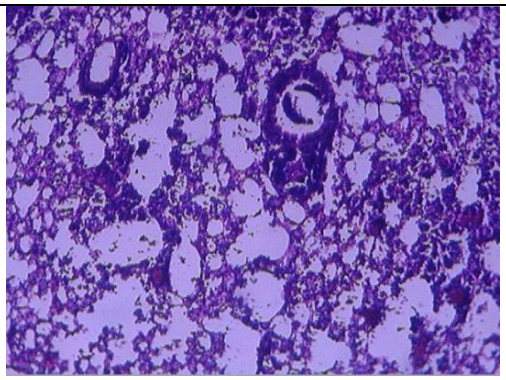
Table 5: Broncho alveolar levage and lung histology in mice

	Group	Eos	Bas	Macrophages /HPF	Epithelial cells
I	Control	7.000 ±0.3162	4.800 ±0.3742	6.200 ±0.3742	6.600 ±0.2449**
II	T1	1.600 ±0.2449**	2.000 ±0.3162**	4.400 ±0.2449**	5.200 ±0.3742**
III	T2	2.000 ±0.3162**	1.200 ±0.2000**	4.600 ±0.2449**	2.200 ±0.2000**
IV	T3	2.800 ±0.2000**	2.000 ±0.3162**	4.000 ±0.3162**	2.000 ±0.3162**
V	T4	3.400 ±0.4000**	3.600 ±0.2449*	3.600 ±0.2449**	3.800 ±0.5831**
VI	T5	3.000 ±0.3162**	1.400 ±0.2449**	4.600 ±0.2449**	3.000 ±0.3162**
VII	Dexamethasone	2.000 ±0.3162**	1.400 ±0.2449**	3.400 ±0.2449**	2.600 ±0.2449*

n = 5; *p<0.05, **p<0.01, compared with control group (ANOVA followed by Dunnett's test)
PMN : Polymorpho nuclear leukocyte
LYM : Lymphocyte
Mon : Monocytes
Eos : Eosinophils
Bas : Basophils

Fig. 2: Histopathological findings of Bronhoalveolar Lavage.

GROUPS	IMAGE	DESCRIPTION
T4		Photograph showing emphysema (yellow arrow), congestion (blue arrow), MNC infiltration (red arrow) and basophils (white arrow); H&E stain 100X

T3		Photograph showing emphysema (yellow arrow), congestion (blue arrow), MNC infiltration (red arrow) and basophils (white arrow); H&E stain 400X
T2		Photograph showing emphysema (yellow arrow), congestion (blue arrow), MNC infiltration (red arrow) and basophils (white arrow); H&E stain 100X
T5		Photograph showing emphysema (yellow arrow), congestion (blue arrow), MNC infiltration (red arrow) and basophils (white arrow); H&E stain 400X
T1		Photograph showing emphysema (yellow arrow), congestion (blue arrow), MNC infiltration (red arrow) and basophils (white arrow); H&E stain 100X

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