

# The effects of salbutamol , methylprednisolone and their combination on the histology of trachea in rabbits

## ABSTRACT

**Background and objectives:** The combinations of a long-acting  $\beta_2$ -agonist to low-to medium doses of inhaled corticosteroids, has become the preferred treatment for moderate persistent and severe asthma in adults and children. This study was conducted to determine the effects of salbutamol, methylprednisolone, and their combination on the histological features of normal tracheal tissue in rabbits.

**Methods:** Forty six rabbits were divided into two groups, control rabbits (n=10) and experimental rabbits (n=36). The experimental group was subdivided equally into three groups that received 0.5mg/kg salbutamol injection for six days weekly, 1.5mg/kg methylprednisolone injection 2 times weekly and combination of the 2 drugs with the same doses and the same time intervals respectively. The samples of the middle part of the trachea were collected 3 weeks and 6 weeks following the beginning of drug administration and the samples were subjected to histological (H&E) and histochemical (PAS stain) procedures.

**Results:** The salbutamol group demonstrated discontinuities among the epithelial cells, an increase in the number of goblet cells together with reduction in the thickness of the smooth muscle. On the other hand, the methylprednisolone group showed metaplasia of the epithelium in many sites together with increase collagen fibers formation in the lamina propria and prominent increased in the number of chondrocytes. The combination of the 2 drugs resulted in limited changes in the epithelium in few regions, with a higher number of chondrocytes when compared with the control group. The intensity of PAS stain was concentrated in the apical cytoplasm of the epithelial cells in the methylprednisolone group and in the perichondrium in the combination group.

**Conclusion:** Salbutamol and methylprednisolone produced many histological changes in the trachea and the combination of the 2 drugs limited these changes.

**Key Words:** Salbutamol, Methylprednisolone  
Histological features, tracheal tissue.

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### Introduction

Salbutamol is used widely for the treatment of bronchial asthma and in obstetrics for the prevention of premature labour (1). The possible clinical side effects after the administration of salbutamol include muscle tremor, palpitations, muscle cramps during intense exercise, and headache (2).

Goblet cell hyperplasia is a prominent feature in animals with atopic asthma and salbutamol enhances goblet cell hyperplasia and airway hyperresponsiveness in rats receiving salbutamol (3). Tamaoki et al. in 2004 stated that the  $\beta_2$ -adrenoceptor receptor agonist stimulates proliferation of airway epithelial cells and produces airway wall thickening in vivo via mitogen activated protein (MAP) kinase-dependent pathway, and these effects are prevented by inhaled corticosteroids (4).

Long-term corticosteroid treatment has been shown to induce pronounced atrophy of the gut and uterine smooth muscle. Furthermore, corticosteroids could affect connective tissue, inducing skin atrophy and marked microvascular changes (5). The anti-inflammatory properties demonstrated for inhaled corticosteroids in biopsy studies on asthmatic patients include reduction in numbers of mast cells, reduction in numbers and activation of eosinophils, reduction in numbers and function of T- lymphocytes, improved epithelial morphology and reduction in epithelial cell activation, and some degree of reversal of airway remodeling (6).

The combinations of a long-acting  $\beta_2$ -agonist to low-to medium doses of inhaled corticosteroids, has become the preferred treatment for moderate persistent and severe asthma in adults and children (7). The early treatment with steroids, alone or in combination with  $\beta_2$ -adrenoceptor agonists, may be highly beneficial to prevent or reduce the extent of airway wall remodeling by limiting inflammation at an early stage. Airway wall thickening, which is largely due to smooth muscle proliferation and hypertrophy, is thought to be an important target for anti-inflammatory drug action. As  $\beta$ -agonists may interact favorably with corticosteroids,

combination therapy may be particularly advantageous in preventing or lessening airway wall remodeling (8).

The beneficial effect of inhaled corticosteroids in the treatment of asthma was studied by Hoshino et al in (1998). They found that 4 months of treatment with inhaled beclomethasone dipropionate (BDP) 500 µg twice daily led to significant reductions in the number of both mast cells and eosinophils, also significant decreases reported in the number of activated eosinophils and mast cells in biopsy samples in the epithelium and lamina propria from patients treated with BDP for atopic asthma (9). These findings confirm the results of earlier studies, in which BDP significantly reduced the number of eosinophils in the epithelium and lamina propria of biopsy samples from asthmatic patients (10).

This study was conducted to determine the effects of salbutamol, methylprednisolone, and their combination on normal tracheal tissue in rabbits as very few studies have dealt with the in vivo effects of these two drugs on normal trachea in rabbits.

## Materials and Method

### Experimental design

The study was performed on 46 male rabbits. Their weight ranged from 1.2-1.8 kg. They were kept in the animal house of the college of medicine /HMU and were fed barley and vegetables in a suitable room temperature (25C).

The rabbits were divided into two groups, experimental (n=36) and control group (n=10). The experimental group was subdivided equally into three subgroups, (12 rabbits for each). The first subgroup received intramuscular injection (IM) of (0.5) mg/kg of salbutamol for six days weekly. The second group received 1.5mg/kg methylprednisolone IM injection twice a week, while the third group received a combination of both drugs in the same doses and time intervals.

Three weeks following the beginning of the experiment, half the number of each experimental subgroup (n=6) rabbits were scarified. Transverse section from trachea of each animal was collected exactly at the middle part of the trachea by using operating scissors. Six weeks following the beginning of the experiment the remaining rabbits of each experimental subgroup were scarified in the same way and a transverse section of trachea was collected from each rabbit. The specimens were kept in 10% formalin for preservation until time of tissue preparation.

Sections of the trachea were also collected from rabbits that didn't receive any type of drugs (control group) in the same way to obtain baseline data of the normal rabbit trachea at light microscopical level. All of the specimens were subjected to Hematoxylin and Eosin PAS stain procedure and examined and photographed using light microscope (Olympus, CH3OR200 Japan and Motic Digital Microscope).

## Results

The histological preparation of the trachea of the control group rabbits showed great similarity to the trachea of the human being. The different levels of the nuclei of the pseudostratified epithelia and the cilia can be easily demonstrated using high power. The goblet cells with their ovoid shape are present between the epithelial cells together with many basal cells. All cells were rested on a well defined thick basement membrane that separate the epithelia from the underlying lamina propria which is consists of loose connective tissue filled with many blood vessels and inflammatory cells, especially lymphocyte (Figure 1 - opposite page).

### Histological findings of 3 weeks salbutamol groups

The injection of salbutamol for three weeks resulted in different changes in the histological appearance of the trachea. Generally the epithelia demonstrate many discontinuities and the underlying lamina propria demonstrated many congested blood vessels. The epithelial tissue of trachea which is usually pseudostratified ciliated epithelia seems to change their appearance approaching simple columnar epithelia with elongated basally located nucleus that were rested on a single squamous basal layer. In addition numbers of goblet cells seemed to be slightly increased; the underlying connective tissue demonstrated many congested blood vessels and reduction of the thickness of the layer as a whole. The basement membrane that separate between epithelia and connective tissue appeared to be obviously thicker than in the control group, (Figure 2 - opposite page).

### Histological finding of 6 weeks salbutamol group

The histological changes that were demonstrated after three weeks injection of salbutamol became more obvious in this group such as the discontinuity of epithelia, the thick basement membrane, and presence of many congested blood vessels. The change of pseudostratified ciliated epithelia to simple columnar epithelia rested on single squamous basal layer became more obvious. The thickness of the underlying lamina propria appeared to be reduced greatly in many regions (Figure 3 - page 28).

The thickness of the trachealis muscle again appeared to be decreased in their thickness especially at the site that devoid the presence of c-shape cartilage, (Figure 4). Using the cytoplasm of the lining epithelia was completely basophilic and the apical portion showed complete positive reaction to PAS stain even for the lining epithelia over the trachealis muscle which resulted in great difficulty in detecting the goblet cells which demonstrated an obvious PAS positive reaction and centrally located nucleus (Figure 5).

### Histological finding of 3 weeks methylprednisolone group

Generally the lining epithelium preserves its pseudostratified columnar feature in many locations while few sites showed the metaplasia of the epithelium to simple columnar to cuboidal epithelium that appeared thinner than in the salbutamol group. The mucosal secretory cells (goblet cells) appeared to increase in number and obviously filled with mucous materials that showed the typical foamy appearance. The underlying lamina propria showed a great reduction in the inflammatory cells

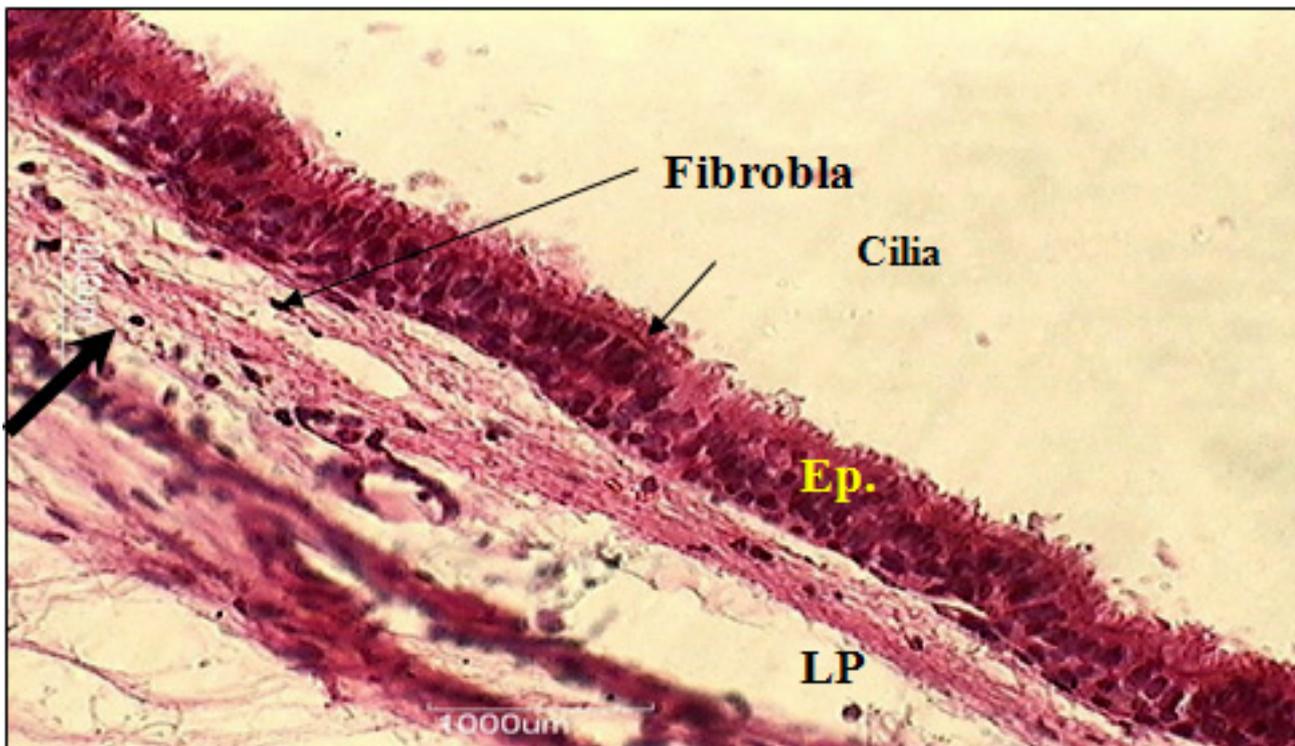


Figure 1 : Photomicrograph of trachea of control rabbit shows the different level of nuclei of epithelial lining (Ep.) and the cilia can be easily demonstrated. Thin collagen fibers are accompanied by fibroblast cells in the underlying lamina propria (LP) together with many lymphocytes (thick arrow). H&E. 400X

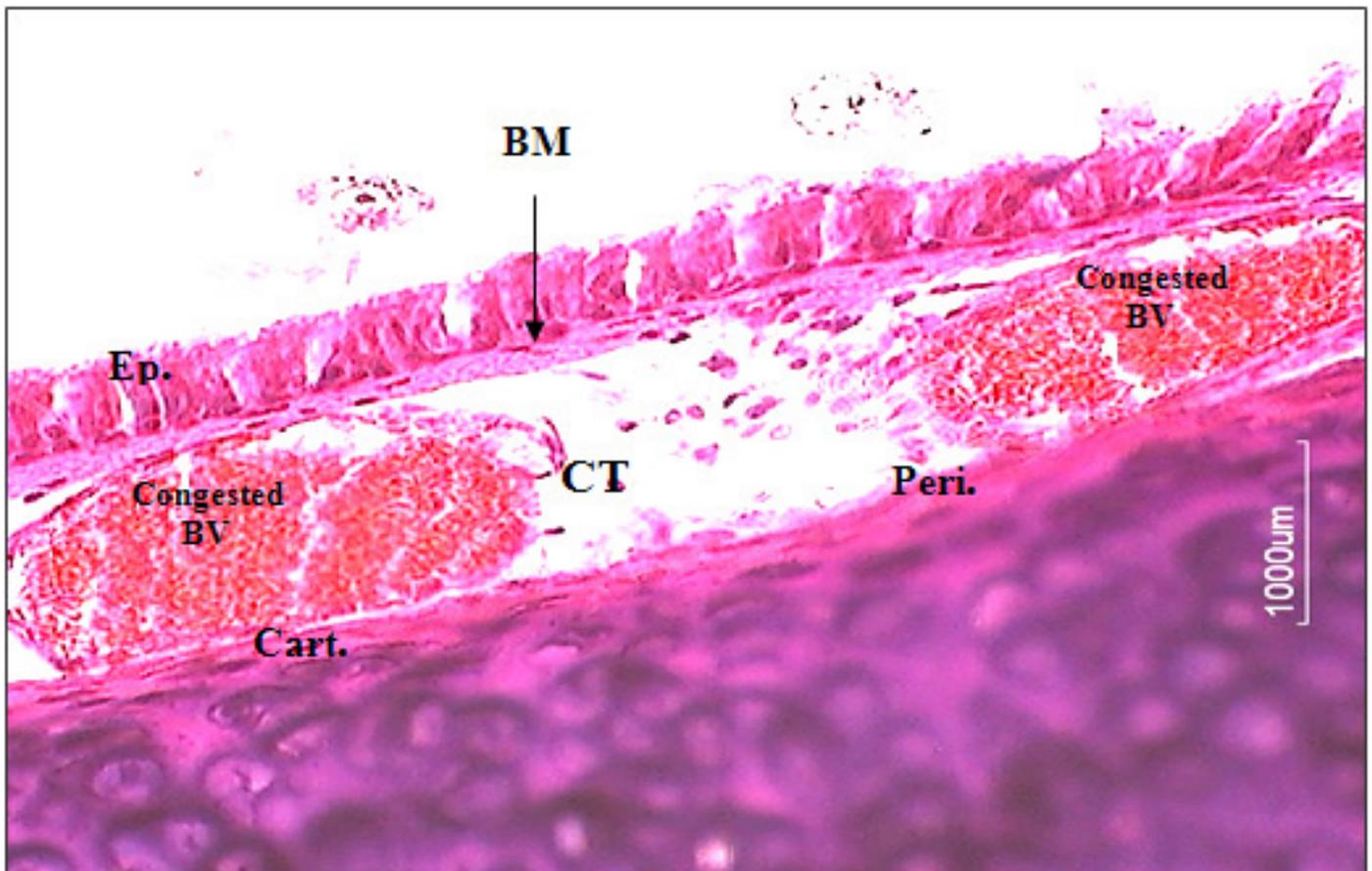


Figure 2 : Photomicrograph of trachea of rabbit 3 weeks after salbutamol injection shows the epithelial discontinuity, underlying connective tissue with many congested blood vessels (BV), and reduction of the thickness of the layers as a whole. The basement membrane (BM) which separate between the epithelium (Ep.) and connective tissue (C.T) appears to be obviously thick. H and E.400X

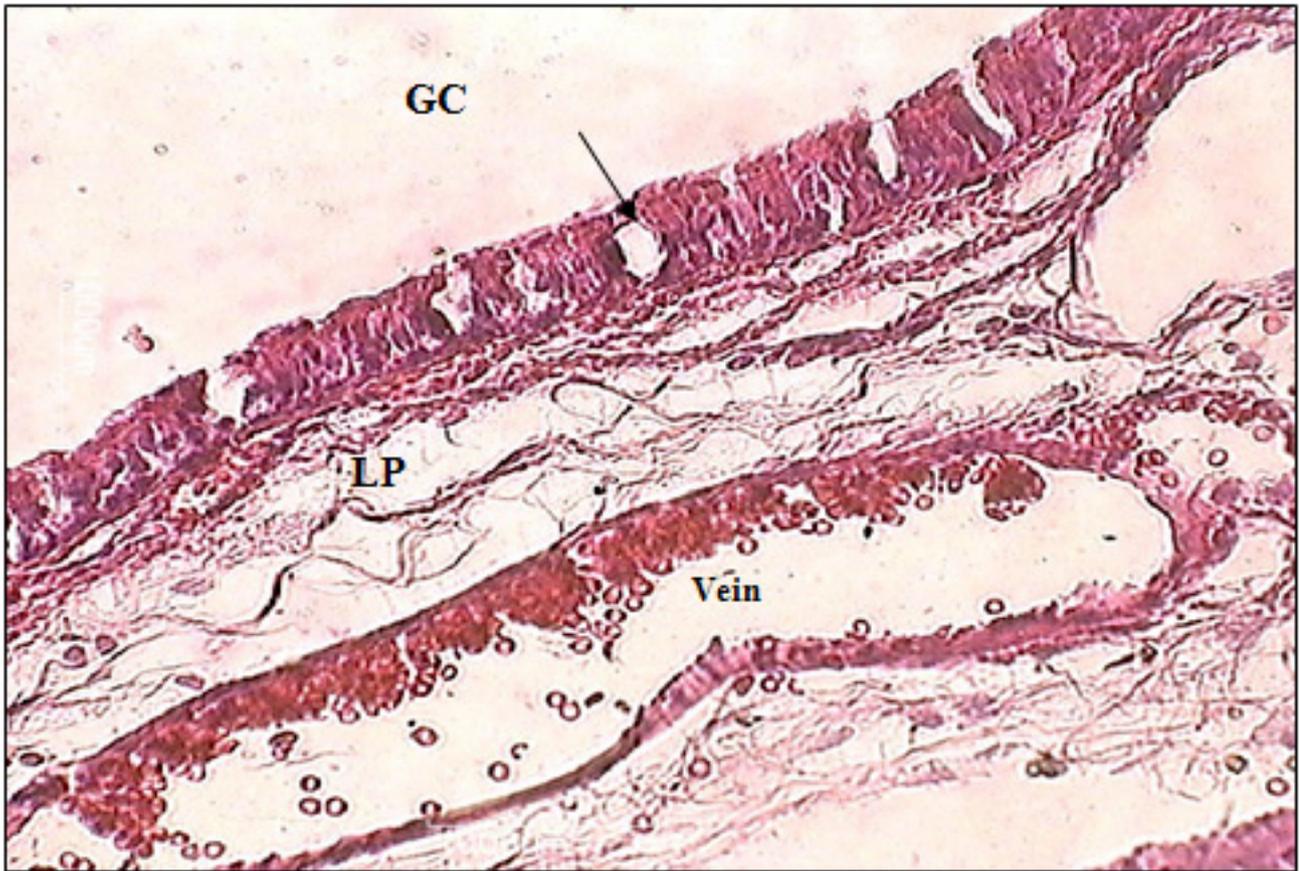


Figure 3 : Photomicrograph of trachea of rabbit 6 weeks after salbutamol injection, shows the discontinuity of epithelium (star) many goblet cells (GC) and many congested blood vessels (BV) in the lamina propria (LP) . H&E. 400X

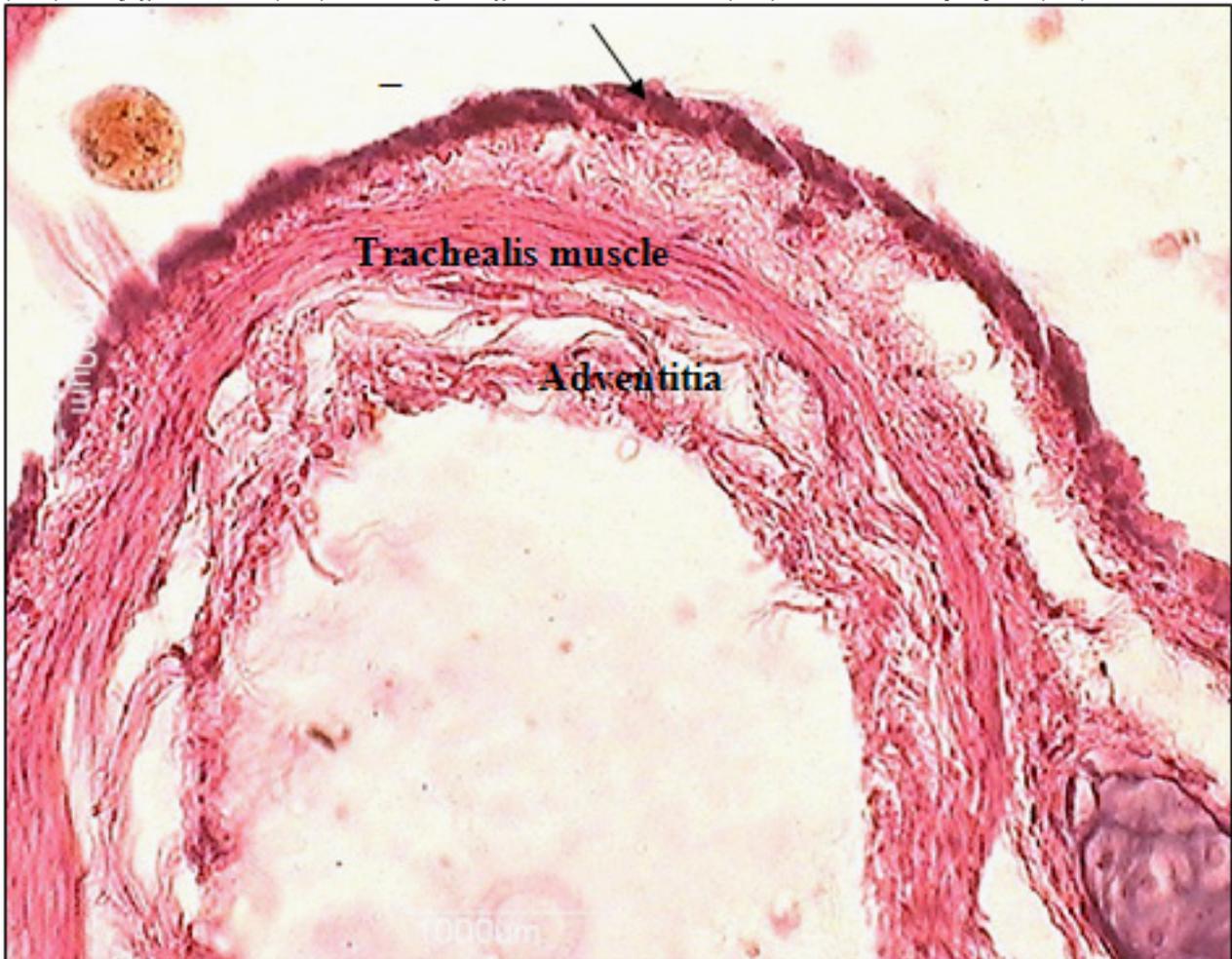


Figure 4: Photomicrograph of trachea of rabbit 6 weeks after salbutamol injection, shows the reduction in the thickness of trachealis muscle. H&E.400X

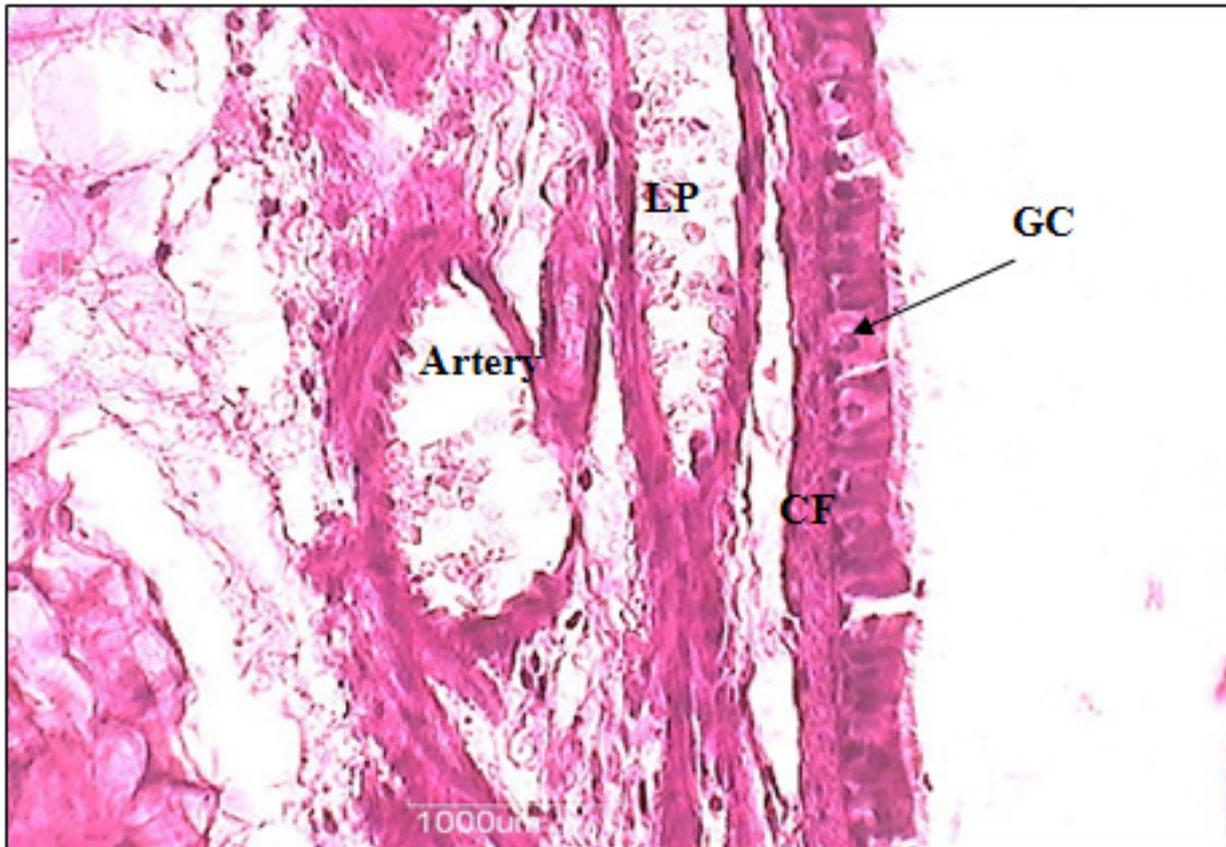


Figure 5: Photomicrograph of trachea of rabbit after 6 weeks salbutamol injection, shows the basal cytoplasm of the lining epithelia is completely basophilic and the apical portion shows complete positive reaction to PAS stain. The nucleus of goblet cells (GC) occupy a central location, the lamina propria (LP) filled with PAS positive congested blood vessels wall. (PAS stain 400X)

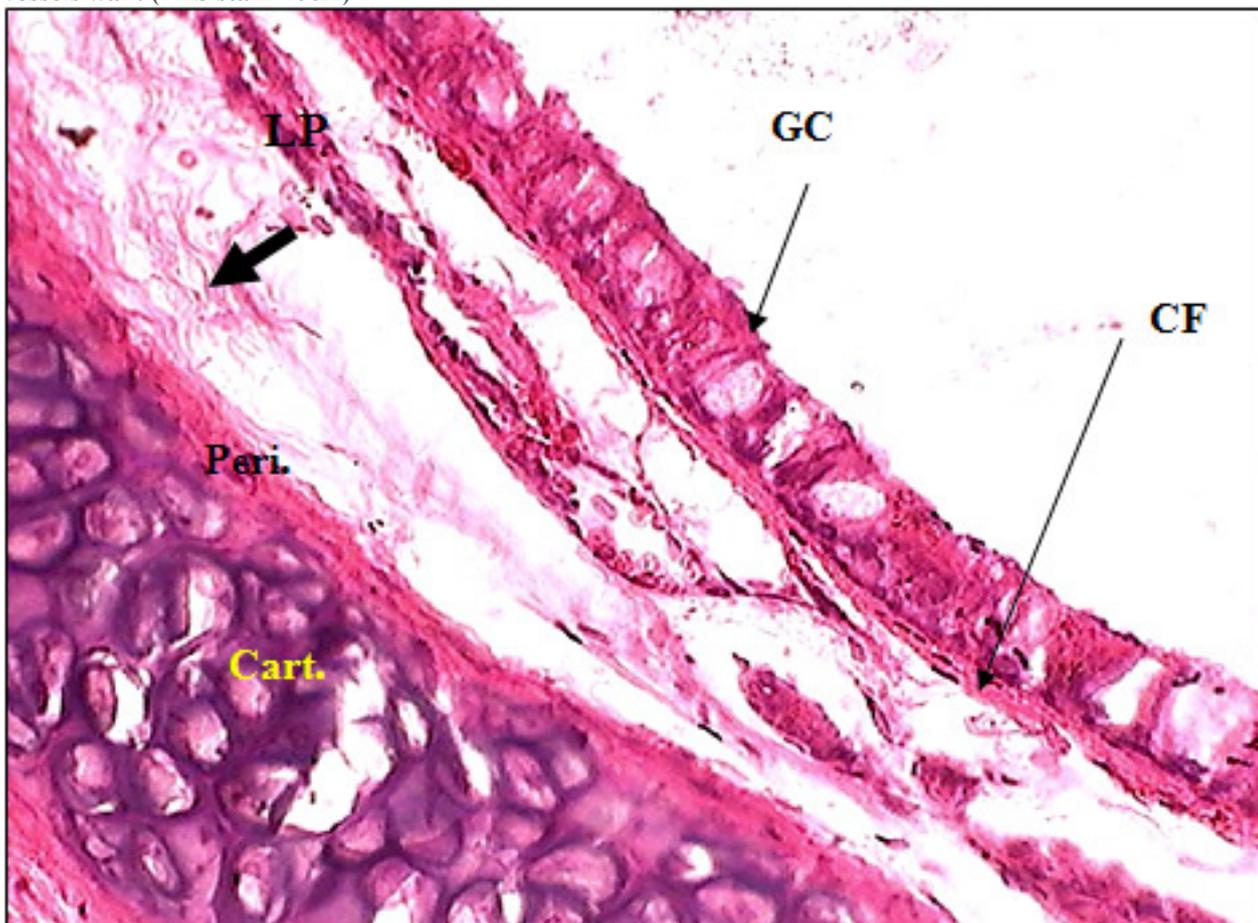


Figure 6 : Photomicrograph of trachea of rabbit 3 weeks after methylprednisolone injection, shows epithelia (Ep.) with many goblet cells (GC) that filled with mucose material and have a foamy appearance. H&E.400X  
BV: blood vessel, Cart: cartilage, CF: collagen fiber, Peri : Perichondrium.



Figure 7 : Photomicrograph of trachea of rabbit 3 weeks after methylprednisolone injection. The chondrocytes in some regions show many opened face nuclei with lack of capsule around the lacunae which indicated low activity in the formation of GAG, Cart: cartilage, Peri: perichondrium. H&E.40

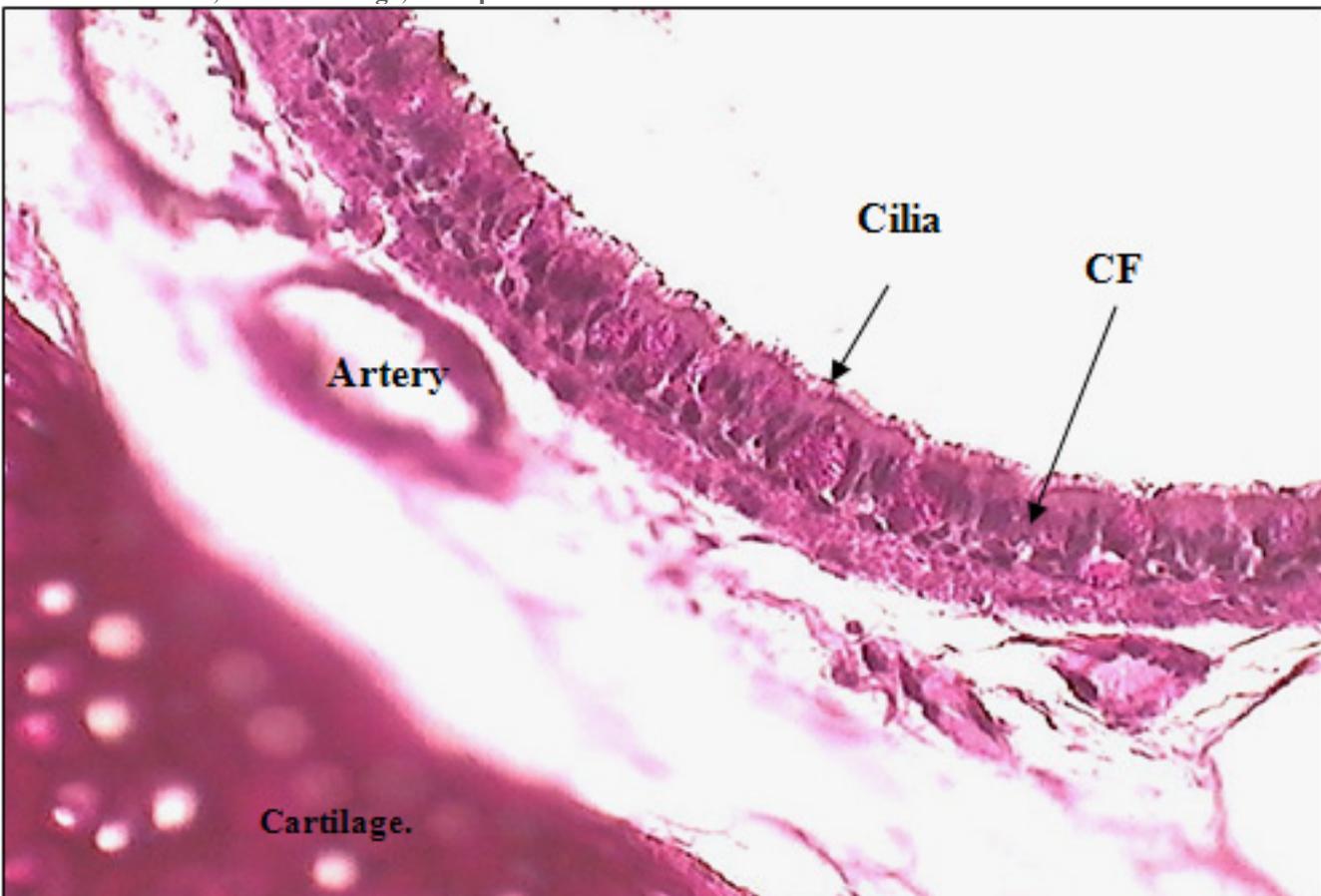


Figure 8 : Photomicrograph of trachea of rabbit after 3 weeks Methylprednisolone injection. The lining epithelia (Ep.) of this group demonstrate the usual pseudostratified columnar epithelia with cilia together with many PAS positive goblet cells (stars), CF: collagen fibers. PAS. 400X



**Figure 9 :** Photomicrograph of trachea of rabbit 6 weeks after methylprednisolone injection. The underlying lamina propria (LP) shows a normal thickness with few inflammatory cells, and a considerable number of cross sections of new blood vessels. (BV) especially within the thick collagen fiber (CF) with budding of new blood vessels (BV), Cart.: cartilage, Peri.: Perichondrium. H&E.400X

(lymphocytes) and an increase in the lay down of collagen fiber. There was a reduction in the number of blood vessels which appeared to have normal wall layers and were not decongestant when compared with the salbutamol group and even when compared with the normal group. Below the epithelium and directly under the basement membrane a thick band of newly formed collagen fiber was demonstrated in different locations (Figure 6). The chondrocytes of the hyaline cartilage showed high degree of mitotic division with the formation of many new isogenous groups which resulted in a crowded appearance of chondrocytes and reduction in the amount of extracellular material. In some regions the chondrocytes show many opened face nuclei with no capsule around the lacunae which indicates high activity in the formation GAG, (Figure 7). The lining epithelia of this group demonstrates the usual pseudostratified columnar epithelia with cilia together with many PAS positive goblet cells that filled with granular materials, (Figure 8).

#### **Histological finding of 6 weeks methylprednisolone group**

The epithelial lining of the trachea of this group showed the histological feature of pseudostratified ciliated epithelia with presence of goblet cells in different sites. The underlying lamina propria showed a normal thickness of loose connective tissue with few inflammatory cells together with the presence of an obviously thick band of collagen fibers directly under the basement membrane and within this band a budding of many newly formed blood vessels were demonstrated (Figure 9).

#### **Histological finding of 3 weeks combination of methylprednisolone and salbutamol group**

Trachea of this group showed some histological differences from the normal group. The epithelia had the normal pseudostratified columnar ciliated appearance which was rested on a thick basement membrane with the presence of many goblet cells among them. As a result of deeply eosinophilic cytoplasm of epithelial cell, the presence of goblet cells become more obvious and their nuclei occupy a central location. The underlying lamina propria which consists of loose connective tissue showed a small number of inflammatory cells when compared with the normal group but at the same time it contained many blood vessels which were congested in many locations but not as in the salbutamol group (Figure 10 - next page).

Generally the PAS reaction in the tissue of the trachea is less intense. The goblet cells can be demonstrated easily since the reaction of the epithelia was less intense and only deep positive reaction was seen in their apical cytoplasm and in their cilia. Using PAS stain the basal cells were completely negative to this stain and were easily demonstrated as a group of cells rested on the basement membrane with slightly wider base than the apex (pyramidal in shape).The underlying lamina propria showed the localization of discontinued collagen fibers directly below the basement membrane. Most of the blood vessels in the lamina propria appeared to be normal with thin walls slightly positive to PAS stain, (Figure 11 - next page).

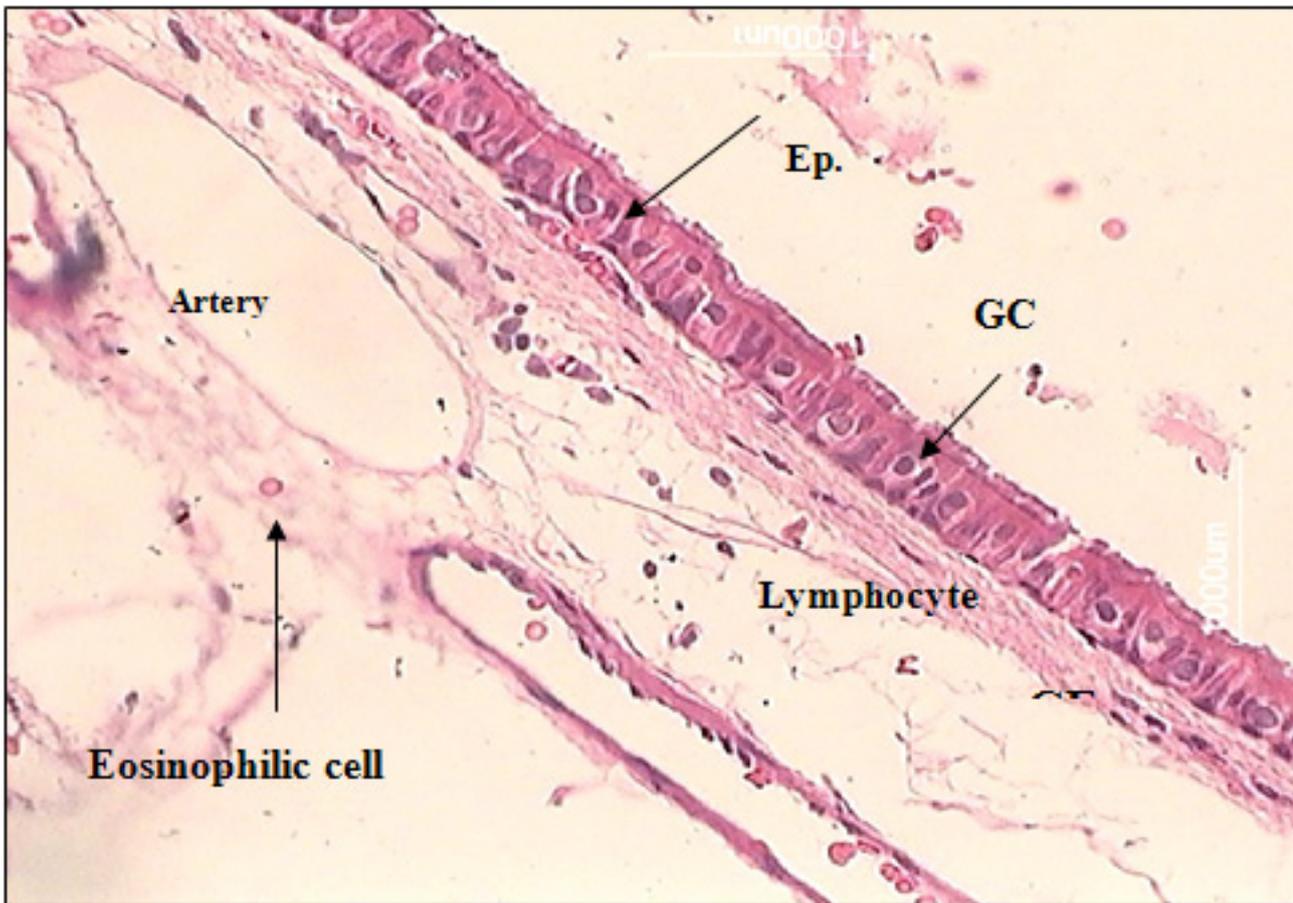


Figure 10 : Photomicrograph of trachea of rabbit after 3 weeks combination of methylprednisolone and salbutamol injection, shows deeply eosinophilic cytoplasm of epithelial cell (Ep), and the presence of goblet cells (GC).Lymphocytes and eosinophilic inflammatory cells were present in the lamina propria (LP) in small numbers with many blood vessels (BV) and thick band of collagen fibers (CF) beneath the basement membrane H&E.400X

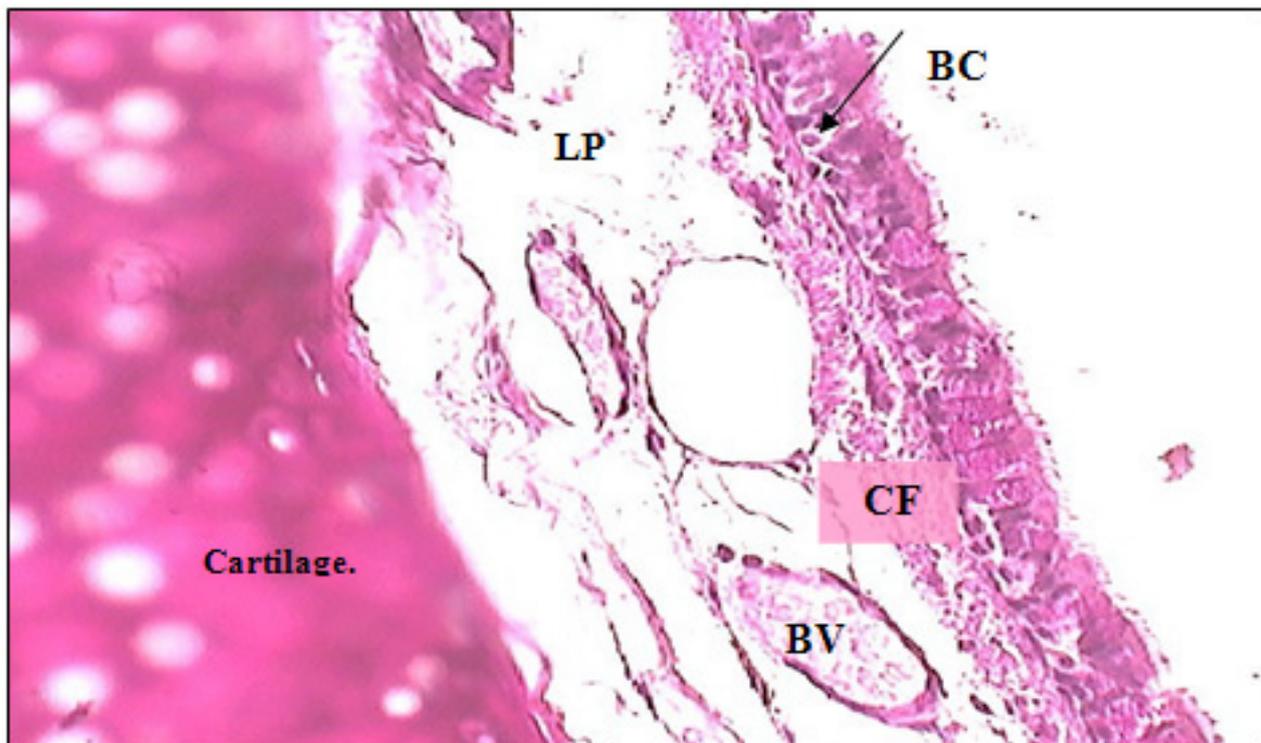


Figure 11 : Photomicrograph of trachea of rabbit after 3 weeks combination of methylprednisolone and salbutamol injection shows the goblet cells (dots), the basal cells (BC) rested on the basement membrane with their pyramidal shape, the discontinuity of collagen fiber below basement membrane, the blood vessels (BV) shows PAS positive reaction in their smooth muscle wall. PAS. 400X,

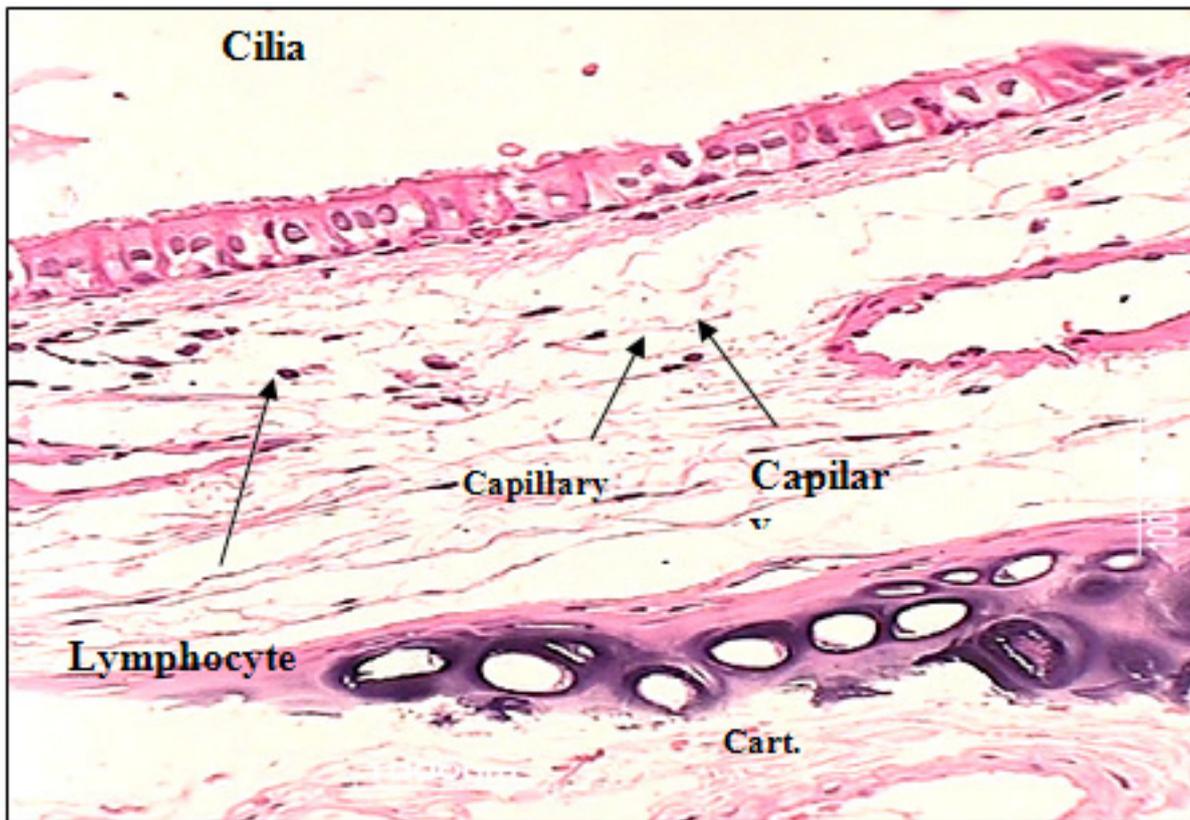


Figure 12 : Photomicrograph of trachea of rabbit after 6 weeks combination of methylprednisolone and salbutamol injection shows many goblet cells (GC) with the nucleus located centrally in most of them, the underlying lamina propria (LP) show the budding of many new small blood vessels (BV) few inflammatory cells. H&E.400X.

#### Histological finding of 6 weeks combination of methylprednisolone and salbutamol group

Pseudostratified ciliated epithelia was detected easily in most of the locations with deeply stained eosinophilic cytoplasm similar to that of 3 weeks combination group. In a few regions, the epithelia seemed to be a single columnar layer that is rested on single squamous epithelia which have flat nuclei resting directly on the basement membrane. The underlying lamina propria showed the budding of many new small blood vessels especially adhering to the basement membrane and generally the lamina propria seems to be highly vascular with inflammatory cells more prominent when compared with the methylprednisolone group, (Figure 12 - above).

#### Discussion

The histological preparation of the trachea of the control group rabbits showed the normal histological features of trachea with great similarity to that of human being. The mucosa consists of pseudostratified columnar ciliated epithelium with many goblet cells that are rested on a thick basement membrane. The cartilage had the typical features of hyaline cartilage with the presence of many isogenous groups of chondrocytes. This histological description was also stated by Vajner et al (2001) who studied the changes of glycoconjugates contained in tracheal goblet cells in rabbits (11).

The injection of salbutamol for three week resulted in different changes in the histological appearance of the trachea. The epithelial tissue of trachea seems to change

their features approaching simple columnar epithelia with the presence of many spacing or discontinuities between the cells, together with a slight increase in goblet cells. In the area of trachealis there was an obvious reduction in the thickness of the smooth muscle. The histological changes that were demonstrated three weeks after the injection of salbutamol became more obvious in the 6 weeks salbutamol group. The thickness of the lamina propria appeared to be reduced greatly in many regions, and the cartilage demonstrated areas of fibrosis in many locations that were devoid of chondrocytes and replaced by a region of highly eosinophilic fibers. These results are in an agreement with Konradova et al (2000) who has studied the trachea of white rabbits 30 minutes after inhalation of 2 puffs of the aerosol of salbutamol. (12).

The results also agree with Kamachi et al (2001) who stated that salbutamol induced more than a two fold increase in the number of goblet cells in rat after 4 weeks of 0.5mg/kg/day salbutamol subcutaneous administration (3). The same histological changes were obtained by Tomlinson et al, (1994) who reported that salbutamol has a direct inhibitory effect on mitogen-induced proliferation of airway smooth muscle cells grown in culture (13), while it disagree with Nishimura et al (2002), who showed that salbutamol stimulates the proliferation of human airway smooth muscle epithelia after it obtained a human bronchial epithelium which incubated with salbutamol for 48 hours in vitro (14). This disagreement could be due to different methods of cells exposure to salbutamol. The result of this study disagrees with Libretto,(1994) who stated that the inhalation of 1000mcg of salbutamol aerosol twice daily for three months did not produce any morphological changes in the lung, trachea of dogs. This

disagreement may be due to the different dose and different way of administration (15).

In the salbutamol group, the lining epithelia showed discontinuity in many locations and the number of goblet cells appeared to be increased and their size appear larger than the control group. In addition, the neighboring epithelia demonstrate PAS positive reaction throughout their cytoplasm. This result is in agreement with the study of Vanjer et.al, (2001) who found that the ultrastructural finding in the tracheal epithelium after the oral administration of salbutamol indicated the overstimulation of the majority of secretory elements resulting even in their damage and degeneration, fifteen minutes post exposure (16).

Histological finding of the 3 weeks methylprednisolone group showed that the lining epithelium preserved its pseudostratified columnar shape in many locations while few sites showed the metaplasia to simple columnar in some locations. The goblet cells appear to increase in number and the underlying lamina propria showed a great reduction in inflammatory cells, and reduction in the number of blood vessels with obvious increase in lay down of collagen fibers. The chondrocytes showed a high degree of mitotic division with the formation of many new isogenous groups and reduction in the amount of extracellular material while the trachealis muscle demonstrated no histological changes.

Methylprednisolone injection for six weeks confirmed the changes which were seen in the three weeks group. In addition the changes became more obvious especially the increase in chondrocyte numbers of hyaline cartilage. This result agrees with Pavlovic et al (1998) who observed that steroid treatment caused a change in the epithelial structure of trachea that appeared monostratified in rats after administration of triamcinolone 1.2mg/kg/day for seven days (17).

The active goblet cells found in this study may agree with the study of Yoshiaki et al (2008) who found that the administration of prednisolone subcutaneously at doses 12.5, 25 and 50mg/kg for 4 days in rats caused increases in the number of goblet cells and mucosal thickening was apparent in the trachea and bronchi (18). The result of this study also agrees with Barnes (2000) who found that the inhaled corticosteroids also improved epithelia morphology and reduced epithelial cell activation with some degree of reversal airway remodeling (19). While our results disagree with Keeley et al (1987) who found that corticosteroid treatment decreases connective tissue synthesis after administration of dexamethasone in rats, this disagreement may be due to the difference in species (20).

The histological findings of 3 weeks combination of methylprednisolone and salbutamol group did not greatly differ from control group. In spite of that, some locations demonstrated areas of variations where epithelial lining appeared to consist of superficial columnar cells and basal cuboidal cells. The underlying lamina propria showed

reduction in the inflammatory cells when compared with the normal group but at the same time it contained many congested blood vessels. The administration of the two drugs for six weeks caused a pronounced increase in goblet cells, the nucleus of which seemed to be located centrally in most of them. The underlying lamina propria showed the budding of much new small blood vessels especially next to the basement membrane with fewer inflammatory cells when compared with control group. These results agreed with the study of Stewart et al, (1997) in which they found that early treatment with steroids in combination with  $\beta_2$ -adrenoreceptor agonists may be highly beneficial in that this approach may prevent or reduce the extent of airway wall remodeling by limiting inflammation at an early stage (8). The results also agreed with the study of Tse et al., (2003) who found that  $\beta_2$ -agonists alone may increase eosinophil survival, whereas corticosteroids reduce survival though opposing effects on apoptosis (21). Similarly  $\beta_2$ -agonists may increase the late response to allergen and the number of eosinophils recruited into the airways, whereas corticosteroids have an apoptosis effect. Thus corticosteroids have the capacity to prevent any potentially adverse inflammatory consequences of chronic  $\beta_2$ -agonist therapy. On the other hand, Orsida et al (2001) showed that the long-term benefits on combination therapy had beneficial effects on the structural changes in the airway that occur in chronic asthma. One of these changes is an increase in airway blood vessels and the combination therapy significantly reduced the number of blood vessels in the airway mucosa (22).

## Conclusion

Salbutamol and methylprednisolone produced many histological changes in the trachea and the combination of the two drugs limited these changes.

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