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The Histological Study to Correlate the Effect of NSAID-Ibuprofen on the Proportion of Epithelial Height in the Wall of Lower Respiratory Airways.

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ABSTRACT

Background: The respiratory system consists of upper and lower respiratory tract. The lower respiratory tract consists of the trachea, principal bronchi and the remaining airway in the lungs. The aim of present study is to correlate the chronic use of NSAID-Ibuprofen (therapeutic doses) and histological changes in mucosa of lower respiratory tract of Swiss albino mice. **Methods:** The adult Swiss albino mice (25 in each group) were given either 40mg/kg commercial ibuprofen suspension (experimental group) or equivalent volume of distilled water (control group) by oral route by gastric gavage method. 7 µm thick longitudinally cut section of lung were studied under microscope after staining with Masson's trichrome, Alcian blue, PAS and H & E stains. Histomorphometry was performed with linear ocular micrometer scale to quantify certain histological parameters namely epithelial height and proportion of epithelium height in an airway wall (PEH). The data was subjected to statistical analysis to obtain significance. **Results:** Proportion of epithelial height in the walls of airways (PEH) was found to be slightly lower in experimental small, intermediate and large airway groups but when compared with control group it was found to be statistically insignificant. **Conclusion:** The apparent decrease in the height of epithelium in the airways of the experimental groups along with the increase in proportion of wall thickness (not significant statistically) is suggestive of a corresponding gain in muscle thickness. This feature might reflect the tendency towards bronchospasm in experimental set of mice.

Keywords: NSAID, Ibuprofen, Proportion of epithelial height, Airway, Histology, Histomorphometric study.

INTRODUCTION

Inflammation is the response of the body to any injurious agent and is characterized by redness, swelling, rise in temperature and pain. Sometimes this inflammatory response is inappropriately triggered or poorly controlled in body and thus responsible for tissue injury. Thus there are many such chronic inflammatory disorders such as rheumatoid arthritis, ankylosing spondilitis, ulcerative colitis and some other nonspecific arthritis which necessitate the intake of anti-inflammatory drugs for a long period of time. Thus in such cases the body is exposed to prolonged use of NSAIDs and thus a person may suffer from the side effects of these NSAIDs.[1]

As analgesics, NSAIDs are unusual in that they are non-narcotic and thus are used as a non-addictive alternative to narcotics. The therapeutic effects of NSAIDs are produced because of their ability to inhibit prostaglandin (PGs) synthesis.[2]

The respiratory tract can be divided into – upper and lower respiratory tracts. The epithelium in the respiratory tract shows gradual decrease in height as we move from upper respiratory tract to lower respiratory tract. In the trachea it is pseudo-stratified columnar epithelium with goblet cells. Slowly as we move downwards it undergoes gradual transition from pseudo-stratified columnar epithelium, columnar epithelium, low columnar epithelium, cuboidal epithelium and finally to simple squamous epithelium in the alveoli.

The alveolar epithelium is a mosaic of two cell types, type I and type II alveolar cells. Type I cells

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form over 90% of the alveolar area although the smaller type II cells are more numerous.

Type I alveolar cells: These are simple squamous epithelial cells which have central nucleus and a highly attenuated cytoplasm about 0.05 to 0.2 microns thick. The edges of the adjacent cells overlap and are adjoined by tight junctions which create a strict diffusion barrier preventing diffusion between the alveolar surface and underlying tissues, a vital feature, which with the similar endothelial barrier, limits the movements of fluid from blood and intercellular spaces into the alveolar lumen. If damaged, the type I cells are replaced by multiplication of type II cells which may latter differentiate into Type I.[3-5]

Type II alveolar epithelial cells: These are less extensive, rounded cells which protrude from the alveolar surface. Their cytoplasm contains abundant mitochondria, granular endoplasmic reticulum, lysosomes and numerous characteristic secretory multilamellar vesicles which contain the precursor of alveolar surfactant. They are more numerous than type I cells but because of their lesser size they form only about 3 % of total alveolar surface. In addition to secreting surfactant components these cells also endocytose and degrade or recycle it.[3-5]

It is well known that NSAIDs have a potentially toxic role on some target areas such as GIT, kidney, internal ear and even CNS. Inspite of rigorous research of literature the published literature stating the effect of Ibuprofen or other NSAIDs on the structure of lung tissue was scanty. The histological study of effect of various drugs on the lung done by taking experimental animals as rat has also been mentioned here.[6,7]

The aim of present study is to co-relate the chronic use of NSAID-Ibuprofen (therapeutic doses) and histological changes in mucosa of lower respiratory tract of Swiss albino mice.

MATERIALS AND METHODS

An experimental set up consisting of twenty five adult Swiss albino mice of either sex in control group as well as in experimental group were used to study effect of chronic use of NSAID-Ibuprofen on histological changes in mucosa of lower respiratory tract.

Drug Ibuprofen (NSAID) in experimental group was administered in the dose of 40 mg/kg and equivalent volume of distilled water in control group of mice, once every day for 6 weeks by oral route using a gastric gavage method.

Light microscope for histomorphometry and self-illuminated trinocular microscope with dimmer and digital camera attachment manufactured by Olympus Company for taking photomicrographs of desired lung fields were used. Linear ocular micrometer and linear stage micrometer were used.

The linear ocular micrometer was calibrated for 4x, 10x, 40x and 100x with the help of stage micrometer. Thereafter this linear ocular micrometer was used as an eye piece in light microscope for histomorphometry.

After completion of 6 weeks of dosage period, animals of control and experimental groups were kept as such for two days and then they were sacrificed within a day.

In the mucosa of lower respiratory tract, the main target area for microscopy in present study was lining epithelium, parenchyma of the lung and any deviation in histo-architecture of mucosal stroma or other components of wall. Most of the microscopic study was performed with H & E stained sections only. This was supplemented by Alcian blue to observe goblet cells but the respiratory tract of mice show negligible number of goblet cells as compared to human lungs. PAS staining was also done when we desired to observe basement membrane of lung alveoli and capillaries. Masson's trichrome stained sections were used specifically to observe distribution of collagen, mucin, smooth muscles, and areas of congested blood vessels in the lung.

The lung airway was examined from without inwards. Firstly we paid attention to the adventitia, cartilage (if present) and smooth muscle, then to the submucosa and lastly to mucosa. The lamina propria was examined in general and then the lining epithelium of respiratory passage was examined. We assessed the relative proportions of epithelial cell height and mucosal thickness under high oil immersion. We also paid attention to any area of fibrosis, haemorrhage, cellular infiltration and deranged architecture anywhere in airway or lung parenchyma.

Height of the epithelium (EH) and Proportion of Epithelial height (PEH)

We measured the height of epithelium at 5 different locations in an airway of interest and finally took the highest epithelial height as standard for that airway. Thus we obtained epithelial height for both the groups. The epithelial height was measured in oil immersion i.e. 100x magnification. Thus the readings of ocular micrometer were multiplied by 1.443 to get readings for epithelial height in microns. PEH refers to the proportion of wall occupied by epithelium.

Overall 100 airways were measured in control as well as experimental group. The data was then arranged in ascending order on the basis of the values of outer diameter. Then these airways were divided in 3 groups on the basis of the outer diameter of the airways. Thus the 3 groups formed were:

1. Airway with small diameter having outer diameter less than 160 microns.

2. Airway with intermediate diameter having outer diameter between 160 to 240 microns.
3. Airway with large diameter having outer diameter more than 240 microns.

The data obtained from control and experimental set of animals was organized under specific heads in different observation tables. The mean values were obtained for any desired parameter using this data and then the statistical significance of the difference in mean value was obtained by applying statistical methods. When number of observations of a desired parameter was less than 30 the comparison was done with unpaired (student's)'t' test. Then a standard value (S) was obtained for 5% level of confidence using d.f. from t-table, which in present study was found to be 2.08. If the calculated difference between mean observed value of a parameter in control & experimental group was more than 2.08 then the change would be considered significant with P value less than 0.05 (with 95% confidence interval). When number of observations of a desired parameter was more than 30 the comparison was done with 'z' test. Here the standard cut off value is 1.96. If the calculated difference was more than 1.96, then the change in parameter would be considered significant with P value less than 0.05 (with 95% confidence interval).

RESULTS

Table 1:Comparison of change in weight of control and experimental groups of mice.

	Control group	Experimental group	P-value
Mean of Change in weight (gm)	-0.32 ± 0.90	-4.31 ± 2.22	P < 0.01***
Mean of Change in weight (percent)	-1.27 ± 3.16	-13.69 ± 6.57	P < 0.01***

*** p< 0.01 highly significant.

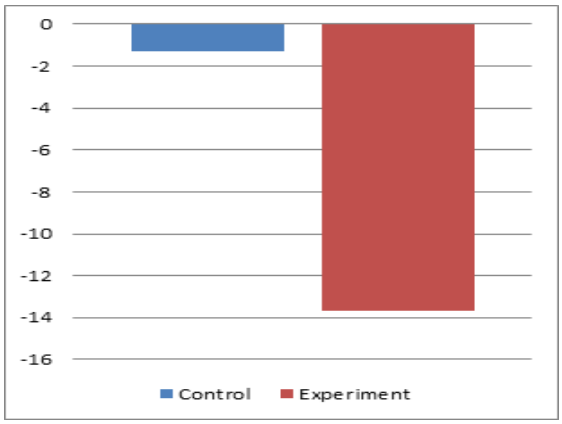

Figure 1: Comparison of change in weight in control and experiment groups of mice shown by bar diagram.

Note: Numbers on Y axis denote values in percent. Negative sign indicates loss of weight.

We measured the weight of mice each time before giving injection to calculate the required dosage daily. The record of initial body weight i.e. at the time of starting the experiment and final body weight at the time of sacrificing the animal was maintained.

The viscera were examined in situ immediately after dissecting the mice before and after perfusion. On gross examination of lungs, both the groups of animals were observed to have no difference. We observed other organs in both the groups. Barring the change associated with sex of the animal, no noticeable change was observed in either group of animals in case of other organs such as liver, kidney, urogenital system and gastrointestinal system. While removing the lung from the thoracic cage of the animal, we didn't observe any abnormal adhesion of the lung with the inner thoracic wall in either group.

Table 2: Comparison on mean PEH value of small airways in both the groups

	Control group	Experimental group	z-value*	p-value
Mean of PEH SD	78.05± 6.20	73.20 ± 5.02	0.00	P > 0.05 N.S

* Cut off value: 1.96 (if z-value < 1.96, then p-value > 0.05 and it is not significant.)

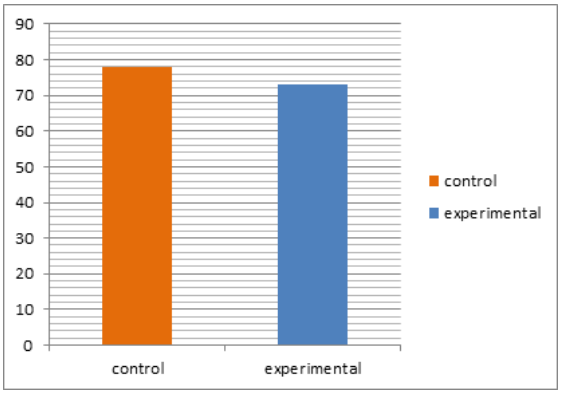

Figure 2: Bar diagram showing the comparison of mean PEH (in %) in control and experimental group-small airways (A).

Note: Numbers on Y axis denote values in percent.

The lung parenchyma did not show much difference in two groups of lungs. The variable expansion of alveoli in different lung fields was almost symmetrical and unremarkable. Alveolar septae were more or less of same thickness in two groups and cellularity of septae was also matching. There was no indication of proliferation or degeneration of pneumocytes I or II. Occasional alveolar macrophages were noted with equal frequency in both groups without there being any

evidence of exudate in alveoli or oedema in alveolar wall. There was also no evidence of fibrosis in Masson's trichrome stained sections or change in thickness of basement membrane of alveoli in PAS stained sections.

Blood vessels throughout the lung stroma were unremarkable and any observable deviation in their appearance was equally seen in both groups of lungs.

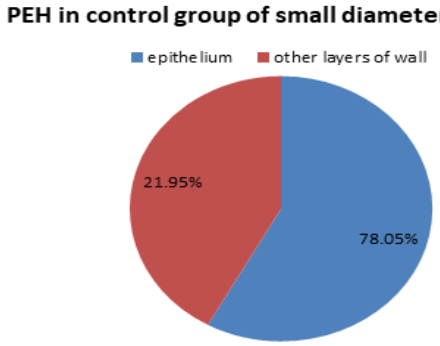

Figure 3: Pie diagram showing the proportion of epithelium in a wall in control group- small airways.

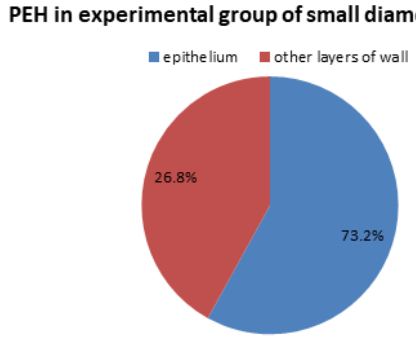

Figure 4: Pie diagram showing the proportion of epithelium in a wall in experimental group- small airways.

Table 3: Comparison on mean PEH value of intermediate airways in both the groups

	Control group	Experimental group	z-value	t-value
Mean of PEH SD	65.56 ± 2.91	63.91 ± 2.62	0.01	P > 0.05

* Cut off value: 1.96 (if z-value < 1.96, then p-value > 0.05 and it is not significant.)

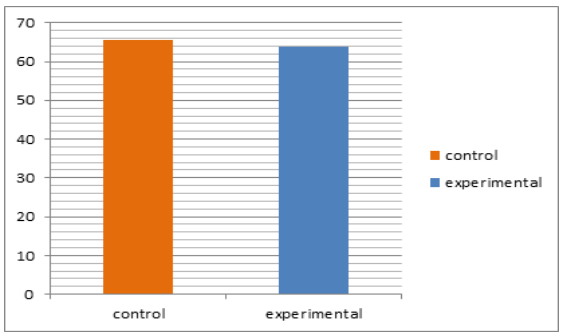

Figure 5: Bar diagram showing the comparison of mean PEH (in %) in control and experimental group- Intermediate airways (B).

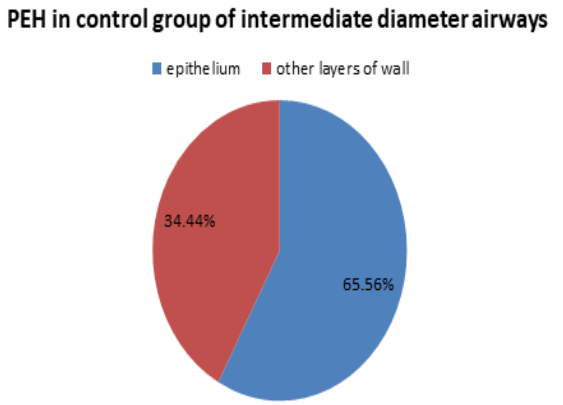

Figure 6: Pie diagram showing the proportion of epithelium in a wall in control group- intermediate airways.

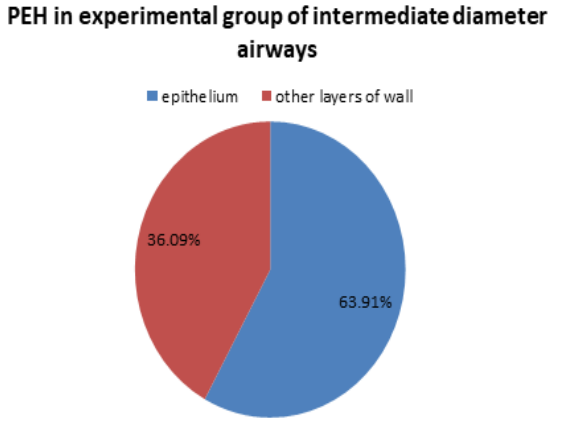

Figure 7: Pie diagram showing the proportion of epithelium in a wall in experimental group- intermediate airways.

Table 4: Comparison on mean PEH value of large airways in both the groups

	Control group	Experimental group	t-value**	p-value
Mean of PEH SD	58.79 ±6.78	58.00 ±6.23	0.36	p> 0.05

**Cut off value: 2.08 (if t-value < 2.08, then p-value > 0.05 and it is not significant)

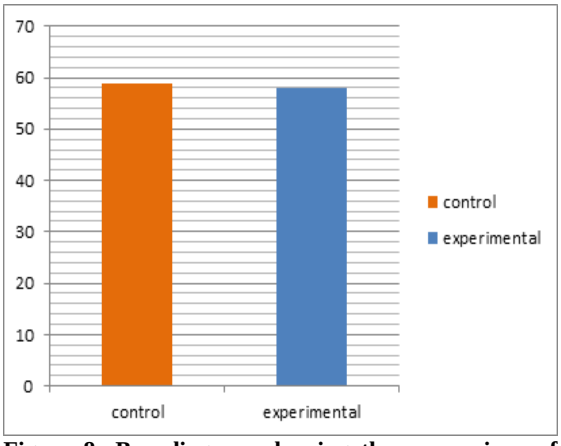

Figure 8: Bar diagram showing the comparison of mean PEH (in %) in control and experimental group- large airways (C).

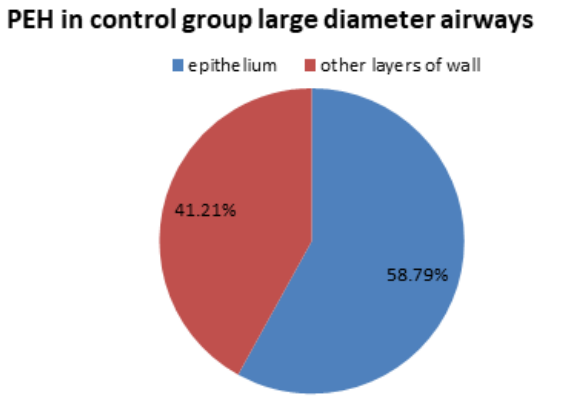

Figure 9: Pie diagram showing the proportion of epithelium in a wall in control group- large airways.

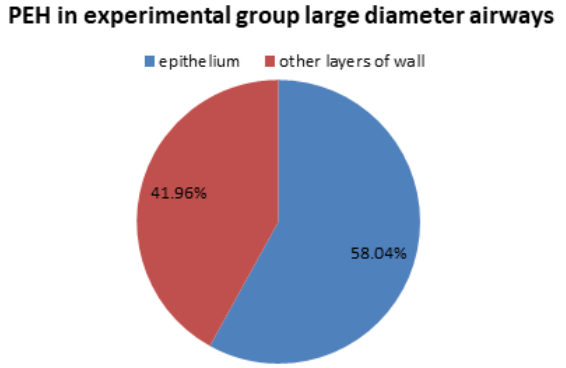

Figure 10: Pie diagram showing the proportion of epithelium in a wall in experimental group- large airways.

DISCUSSION

Several factors which favour inclusion of this species for animal experimentation are easy availability, short gestational period, large litter size, ease to handling due to docile nature and their easy adaptability to unfamiliar geographic conditions. Dudkiewicz J and Ettarh have also used mice for their experiments related to NSAIDs.[8,9] Fehrenbach H and Gad S.C used rats in their experiments on NSAIDs even though rats are more aggressive than mice.[10,11] Gumuser G used horse as experimental animal for carrying out a study of effects of NSAIDs.[12] Halford GM has mentioned that the drug dose and pharmacokinetics of the NSAIDs is different for different species.[13] They used rats, dogs and monkeys in their experimentation. We didn't take the pharmacokinetics of mice into account due to the ease and safety of usage of Swiss albino mice.

In present study we have administered drug ibuprofen at a dose of 40mg/kg i.e. 0.04 mg/gm body weight daily for 6 weeks. The therapeutic dose for humans is 8-10 mg /kg per dose given 3-4 times a day according to standard literature.[14] Thus it can be concluded that the equivalent single dosage per day for mice without toxicity would be 40mg/kg.

In our study drug was given by gastric gavage method with the help of feeding cannula attached to syringe. Husain AN and Kim JY have also use gastric gavage method for drug administration in their experiments.[15,16] We preferred gastric gavage method as we could avoid the spillage of drug that could occur by oral route. Secondly we could offer a standardized drug dose of ibuprofen to mouse by taking into consideration its body weight. When oral dosage of drug is administered by mixing it in food or water there would be subjective variation in the dose administered to different mice in same experimental setting. Thus we avoided the latter method as it could affect the results.

In our study ibuprofen was administered to the non-fasting and non-sedated animal. The mice had free access to food and water. They were given exposure to standard climatic conditions including temperature, light and humidity. Many studies have also given doses to non-fasting animals similar to our method.[17-20] Some researchers used fasted animals for dosing of NSAID.[21-23]

We believe that the method of sacrificing by cervical dislocation without any premedication used by us is the most suitable method of sacrifice. We believe that this method neither leaves any possibility of interference of any other drug to produce changes in any organ systems of mice nor does it affect the lung architecture as there are no chances of hyperventilation or petechial haemorrhages in mice by this method.

During dosing period no change in animal behaviour was observed. The animals were procured from professional breeders for experimentation in our institutional animal house. We kept them under observation for one week after bringing them. Thereafter we initiated the drug treatment.

During this period we observed that both the group of mice had a dislike for the food provided in the cage. This could be possibly due to change in their surroundings, quality of food and living space. We used 25 animals in each group. Although the fierce fights amongst the animals living in same cage and the resultant deaths are not uncommon in experimental settings, in our study no death was reported in our study in either of the two groups.

The proportion of epithelial height (PEH) with respect to wall thickness in a given airway suggest us the % share of epithelium in the composition of the wall and therefore the % share of rest of the elements in the wall other than epithelium. In present study the parameter of PEH was found to be apparently reduced in all three experimental airway groups (small, intermediate and large) but the reduction was not statistically significant. Since the cartilage is largely missing from all intrapulmonary airways in mice as we also observed it, the sub epithelial lamina propria was hardly ever measurable in greatest possible

magnification of light microscope so the only other element of the wall which could make a difference in wall thickness other than epithelium, was smooth muscle layer. Since PWT was unaltered and the PEH was apparently reduced in experimental group, it could be assumed that there was a corresponding gain of muscle element in the wall of airways. In absence of a visible alteration in layers of muscle in airways the slight change in the wall thickness could be brought by an increased width of smooth muscle cells due to increased tone (spasm). Thus it can be interpreted that the slight change which we observed in lungs of experimental mice was that of the spasmodic state of airways, especially of small and intermediate diameter and that was not accompanied by any concurrent inflammation by and large. However such change was only apparent and there was no statistical significance. Bayomi et al (2008) has reported increase in thickness of alveolar septa along with mononuclear cell infiltration and alveolar damage with collapsed alveoli in the albino rats treated with Leflunomide- an immune modulator and anti-inflammatory drug given for rheumatoid arthritis. They have reported the changes to be reversible. They used electron microscopy and immune-histochemistry (caspase-3 antibody for detection of apoptotic cells.) we had limited scope of light microscopic observations in present study. So such parameters could not be ascertained in present study. Further in routine H&E stained sections, alveolar septae differed markedly in thickness in different microscopic field of the same lung tissue and it became very difficult to attempt for measuring thickness of alveolar septae by morphometry. However quantatively we did not find any noticeable change in cellularity (both type I or type II pneumocytes) in alveolar septae, nor there were evidences of collagen deposition in the alveolar wall in Masson's trichrome stained sections. There was no thickening of basement membrane in PAS stained sections. There was no evidence of cellular infiltration in alveolar stroma or lumen. So the present study does not reflect changes observed by Narayanankutty A.[24] The sporadic areas where vessels were found engorged in the stroma or where the several alveoli appeared almost collapsed in the lung fields, were not uniformly associated with any group of animals. So the feature has been ignored.

In the lung of two animals of the experimental group there was evidence of dense mononuclear cell infiltration in stroma and large aggregates of lymphocytes here and there. Again this was considered as a chance occurrence, probably because the animals might have caught respiratory infections at the time of sacrifice and such features were considered unworthy of reporting in present study.

The apparent reduction in the height of epithelium along with the increase in proportion of wall thickness in the airways of the experimental group is suggestive of a corresponding gain in muscle thickness. This feature might reflect the tendency towards bronchospasm in experimental set of mice though not that remarkable so as to cause any gross histological change in the lung structure.

CONCLUSION

1. Reduction in the body weight at the end of dosing period in both the groups of mice but significant weight loss in experimental group of mice.
2. There was apparent reduction in the height of the epithelium in the airways of the experimental group though statistically insignificant.
3. Proportion of epithelial height in the wall of airway (PEH) was found to be slightly lower in experimental small, intermediate and large airway group but when compared with control group it was found to be statistically insignificant.
4. The apparent reduction in the height of epithelium in the airways of the experimental group along with slight increase in proportion of wall thickness is suggestive of a corresponding gain in muscle thickness. This feature might reflect the tendency towards bronchospasm although the findings were not statistically significant.

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