

EFFECT OF SEX DIFFERENCE ON THE HISTOLOGICAL STRUCTURE OF BRONCHUS AND NASAL CAVITY IN ANIMAL ASTHMA MODELReinaldi Rachmadhianto¹, Tri Hartini Yuliawati², Gatot Soegiarto³¹Medical Study Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia²Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia³Division of Allergy & Clinical Immunology, Department of Internal Medicine, Dr. Soetomo General Hospital, Surabaya, Indonesia**ABSTRACT**

Background: *The prevalence of asthma in prepuberty woman is more common than in puberty men. However, after puberty to a certain age, women dominate more. It is still unclear whether sex hormones affect the histological structure of male and female airways or not.* **Objective:** *This study objective is to examine the effect of sex difference on the histological structure of bronchial and nasal cavity of mice model (*Mus musculus*) with ovalbumin exposure.* **Material and Method:** *This study used 24 mice in four groups (male control, female control, male asthma model, and female asthma model). At the sensitization phase in days 0 and 14, mice were injected intraperitoneally with 100 μ l of a mixture of 50 μ l ovalbumin (200 μ l / ml) and 50 μ l alum. At the exposure phase in days 21 to 23, mice were exposed to 1% ovalbumin (aerosol, 30 minutes / per day). Mice were sacrificed 48 hours after the last exposure. The data taken included four variables, namely: bronchial epithelial thickness, bronchial smooth muscle thickness, nasal cavity goblet cells number, and nasal cavity mucosal thickness. Next, Shapiro-Wilk normality test and parametric t-test were conducted.* **Result:** *In animal asthma models, mice with male and female exposure did not cause differences in epithelial thickness and smooth muscle thickness in bronchus compared to the control group. However, there were significant differences in the number of goblet cells and mucosal thickness in nasal cavity of male and female mice compared to the control group (respectively, $p = 0.002$; $p = 0.006$ and $p = 0.003$; $p = 0.005$). There were no significant differences between groups of male and female mice on all variables.* **Conclusion:** *In animal asthma models of mice, ovalbumin exposure did not cause differences in the values of all variables between male and female groups.*

Keywords: *Asthma, sex difference, respiration, airway*

Correspondence: Tri Hartini Yuliawati, dr., M.Ked., PA, Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Jl. Mayjen. Prof. Moestopo no. 47, Surabaya 60131, East Java, Indonesia, trihartini77@yahoo.co.id

Background

Asthma is a heterogeneous disease that is usually characterized by chronic airway inflammation, a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and coughing whose intensity varies from time to time and accompanied by varying expiratory airflow obstructions (Global Strategy for Asthma Management and Prevention, 2018). In general, at the age of children or pre-puberty, asthma attacks more men than women, but after the age of pre-puberty asthma attacks more women, both in the community and who come to the hospital due to acute asthma attacks (Singh, *et al.*, 1999; De Marco, *et al.*, 2000; Schatz, *et al.*, 2006; Vink, *et al.*, 2010).

It may be that the impact of standard therapy is not significantly felt by the difference in female or male asthma patients, but epidemiological data show that adult women suffer more and are more likely to suffer from an asthma exacerbation than men (Schatz, *et al.*, 2006). The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) and the Severe Asthma Research Program (SARP) found that women had 4.4 times more severe asthma than men (Abraham, *et al.*, 2003; Moore, *et al.*, 2010). Exacerbation of asthma during pregnancy is a significant problem and can have a devastating effect. During pregnancy, 20% of women experience exacerbations, and 6% require hospitalization (Murphy, *et al.*, 2006). Women with asthma who experience exacerbations during high-risk pregnancies give birth to babies with the low birth weight with all the associated effects (MacMullen, *et al.*, 2010; Vanders & Murphy, 2015).

The difference in the incidence of asthma by sex difference cannot be fully explained, but the transition to asthma incidence after puberty implies a hormonal influence. Asthma symptoms in women seem to be influenced by several phases of life, such as menstruation, pregnancy, and menopause (Farha, *et al.*, 2009). Airway sensitivity in asthma patients is usually measured by the provocative concentration of methacholine needed to cause a 20% decrease in FEV1 from basal (PC20). Adult women with controlled asthma experience a decrease in PC20 of more than half during the menstrual cycle, the lowest PC20 value occurs at peak concentrations of estrogen and progesterone in the luteal phase

(Tan, *et al.*, 1997). The PC20 change cycle is believed to be caused by abnormal adrenoceptor β_2 control in the premenstrual period and is thought to be influenced by sex-steroid hormones (Wheeldon, *et al.*, 1994; Wei *et al.*, 2015). It is still unclear whether progesterone or estrogen levels or a balance between the two can cause worsening asthma symptoms (Zein & Erzurum, 2015), but several new studies have shown that endogenous sex hormones can indeed affect lung function and asthma control. Estrogen or estradiol is known to increase Th2-mediated inflammation in the airways after allergen exposure. Estrogen also plays an important role in the secretion of IL-17A, which leads to airway inflammation (Fuseini & Newcomb, 2017; Yung, *et al.*, 2018).

From this description, it is necessary to prove whether estrogen is indeed a determining factor in the difference in the incidence of asthma by sex difference. Given its effect, estrogen can affect the structure or anatomy of the airways (ie affect the number of goblet cells and trigger goblet cell metaplasia, which may also contribute to thickening of the airway mucosa) which will ultimately affect the incidence and severity of asthma symptoms in adult women. To prove this, an invasive examination is needed, which is an airway biopsy in female patients with severe asthma, bronkoalveolar fluid rinses, pulmonary examination and blood tests are also needed. Invasive examination is certainly constrained technically and ethically, therefore research is needed by modeling asthma in experimental animals. Among the several animal models, the asthma model in mice is one of the main choices because mice have a very homologous immune system with humans, the technique is relatively easy and tested, and various commercial reagents are available on the market (Hellings & Ceuppens, 2004; Aun *et al.*, 2017; Haspeslagh *et al.*, 2017). Researchers will evaluate the histological differences in male adult mice, female adult mice that are sensitized and exposed to inhalation allergens, and compare them with control groups that are not treated.

Objective

In general, the objective of this study is to explain the differences in the incidence of asthma in women and men through proving the presence of differences in microscopic

structures in the bronchial and nasal cavity of the mice (*Mus musculus*) asthma model with ovalbumin exposure in male and female mice. However, specifically, the objectives of this study were four points:

1. Proving the differences in the thickness of the epithelium and smooth muscle in the bronchus of mice (*Mus musculus*) asthma models that are exposed to ovalbumin.
2. Proving the difference in the number of goblet cells and thickness of mucosa in the nasal cavity of mice (*Mus musculus*) asthma models that are exposed to ovalbumin.

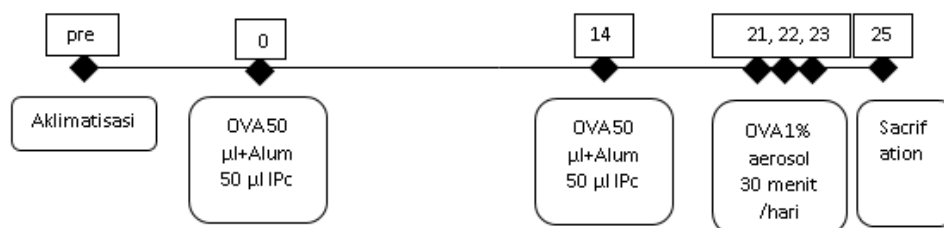
Material and Method

This research is an experimental study with a post-test only control group design. Inclusion criteria include BALB / c mice (*Mus musculus*) 6-8 weeks old weighing 20-40 grams (male) and 18-35 grams (female),

healthy condition, and active movements. Exclusion is done if there are mice that experience pain that is not caused by the research treatment and are thought to affect the observed variables.

Experimental animals will be divided into four groups (male control, female control, male treatment, female treatment). The exposure of ovalbumin is done in two stages, namely sensitization, and challenging. In the sensitization stage, the treatment group mice will be injected intraperitoneally on days 0 and 14 with 100 μ l mixture consisting of 50 μ l ovalbumin (200 μ l / ml) and 50 μ l alum. In the challenging stage, mice will be exposed to aerosol 1% ovalbumin for three days, starting from day 21 with the duration of 30 minutes of exposure per day. Then the airway preparation will be taken 48 hours after the last exposure (Wagers et al., 2007).

Figure 1. Method's Flowchart



The normality of the data distribution was tested using the Shapiro-Wilk test. While different variables were tested using the independent sample t-test. Data were analyzed using legal SPSS software. This study was approved by the Ethics Committee of the Medical Research Ethics Committee of the Airlangga University Medical School.

Results

In female mice, the epithelial thickness increased from $18.962 \pm 2.23 \mu\text{m}$ to $20.287 \pm 2.6 \mu\text{m}$ ($p = 0.370$). In male mice, smooth muscle thickness increased from $15.219 \pm 4.25 \mu\text{m}$ to $18.596 \pm 2.45 \mu\text{m}$ ($p = 0.152$). There was no significant difference in the thickness of the bronchial epithelium in male and female mice in the asthma model mice ($p = 0.303$). The results can be seen in **table 1**, while the histological picture can be seen in **figure 2** and **figure 3**.

In female mice, smooth muscle thickness increased from $10.138 \pm 1.75 \mu\text{m}$ to $12.268 \pm 4.19 \mu\text{m}$ ($p = 0.277$). In male mice, an increase in the thickness of smooth muscle occurred from $7.048 \pm 1.8585 \mu\text{m}$ to $11.142 \pm 2.18 \mu\text{m}$ ($p = 0.015$). There was no significant difference in bronchial smooth muscle thickness in male and female mice in the asthma model mice ($p = 0.602$). The results can be seen in **table 1**, while the histological picture can be seen in **figure 2** and **figure 3**.

In female mice, the number of goblet cells increased from 2.167 ± 1.59 to 5.833 ± 1.51 ($p = 0.006$). In male mice, an increase in the number of goblet cells from 1.958 ± 1.44 to 4.583 ± 1.18 ($p = 0.002$). There was no significant difference in the number of nasal cavity goblet cells between male and female mice in the asthma model mice ($p = 0.140$). The results can be seen in **table 2**, while the histological picture can be seen in **figure 4** and **figure 5**.

In female mice, the thickness of the mucosa increased from $26.502 \pm 1.68 \mu\text{m}$ to $43.511 \pm 9.01 \mu\text{m}$ ($p = 0.005$). In male mice, the mucosal thickness significantly increased from $27.147 \pm 3.55 \mu\text{m}$ to $40.479 \pm 7.83 \mu\text{m}$ ($p = 0.003$). There was no significant difference in

mucosal thickness of the nasal cavity between male and female mice in the asthma model mice ($p = 0.548$). The results can be seen in **table 2**, while the histological picture can be seen in **figure 4 and figure 5**.

Table 1. Epithelial Layer and Smooth Muscle Layer Thickness on Bronchus

	Epithelial Thickness (μm)	Smooth Muscle Thickness (μm)
Male Asthma Model		
Control	$15,219 \pm 4,25$	$7,505 \pm 1,85$
Treatment	$18,596 \pm 2,45$	$11,142 \pm 2,18^*$
Female Asthma Model		
Control	$18,962 \pm 2,23$	$10,138 \pm 1,75$
Treatment	$20,287 \pm 2,64$	$12,268 \pm 4,19$
Comparison of Asthma Model		
Male Treatment	$18,596 \pm 2,45$	$11,142 \pm 2,18$
Female Treatment	$20,287 \pm 2,64$	$12,268 \pm 4,19$

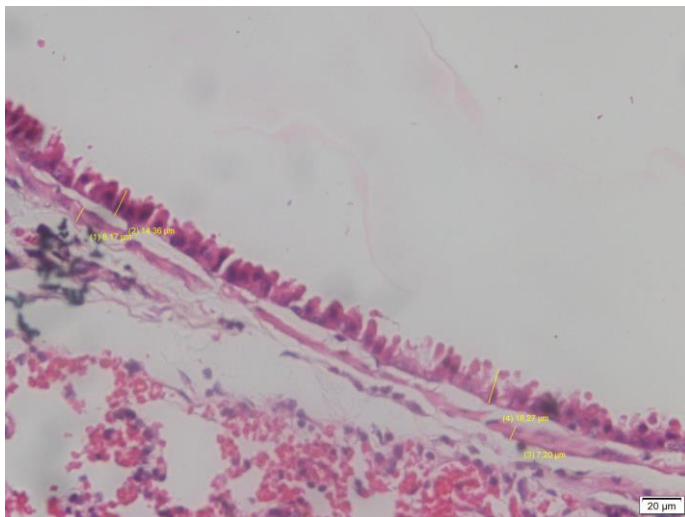
* $p < 0,05$ = significant value $p > 0,05$ = insignificant value

Table 2. Goblet Cells Number and Mucosal Layer Thickness on Nasal Cavity

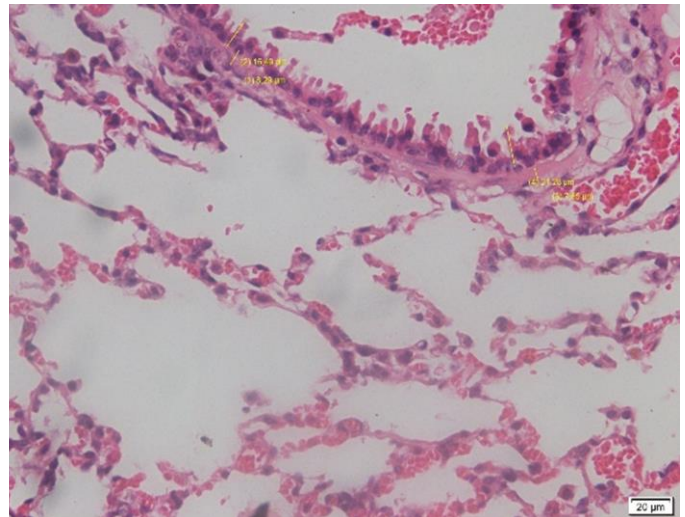
	Goblet Cells Number (per $200\mu\text{m}$)	Mucosal Thickness (μm)
Male Asthma Model		
Control	$1,958 \pm 1,44$	$27,147 \pm 3,55$
Treatment	$4,583 \pm 1,18$	$40,479 \pm 7,83$
Female Asthma Model		
Control	$2,167 \pm 1,59$	$26,502 \pm 1,68$
Treatment	$5,833 \pm 1,51$	$43,511 \pm 9,01$
Comparison of Asthma Model		
Male Treatment	$4,583 \pm 1,18$	$40,479 \pm 7,83$
Female Treatment	$5,833 \pm 1,51$	$43,511 \pm 9,01$

* $p < 0,05$ = significant value $p > 0,05$ = insignificant value

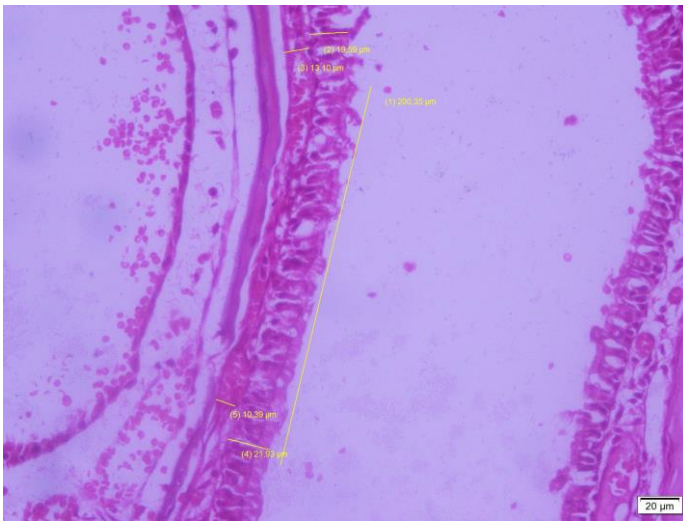
Gambar 2. Bronchial Histology of Male Mice with HE Staining.



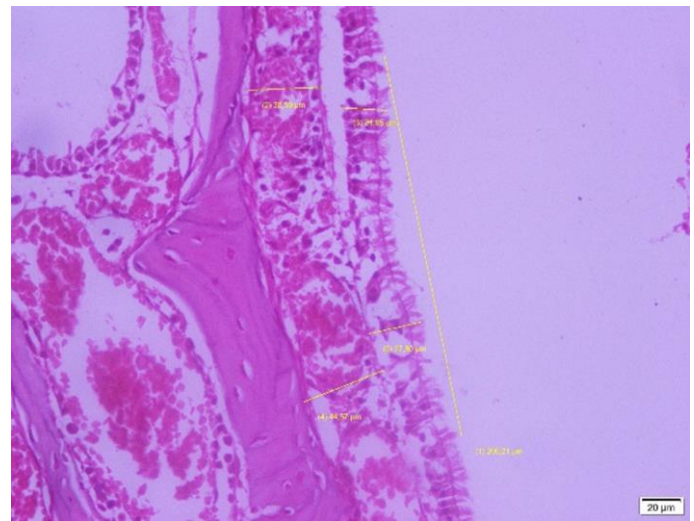
Gambar 3. Bronchial Histology of Female Mice with HE Staining.



Gambar 4. Nasal Cavity's Histology of Male Mice with HE Staining.



Gambar 5. Nasal Cavity's Histology of Female Mice with HE Staining.



Discussion

Chronic Th2 type inflammation in the airways can cause structural changes in the airway mucosa and activation of various immune cells, which will eventually cause deterioration of the lung physiology over time. Most immune cells express estrogen receptors ($ER\alpha$, $ER\beta$, or membrane-bound G-protein-coupled ER) and can, therefore, respond to these hormones. Through its interaction with these receptors, estrogen can regulate the differentiation and activation of several immune cells (macrophages, dendritic cells, eosinophils, mast cells, airway epithelial cells, T lymphocytes and B lymphocytes) including their migration processes, survival, cytokine production and antibodies (Keselman & Heller, 2015). Estrogen has been shown to increase airway sensitivity to allergen exposure (Matsubara, *et al.*, 2008). Through Th2 type inflammation, estrogen indirectly plays a role in goblet cell metaplasia in the airways (Boucherat, *et al.*, 2013). In addition, estradiol supplementation was also able to increase the number of goblet cells and trigger mucous synthesis in the airway epithelium (Tam, *et al.*, 2014). Thus, estrogen is reasonably suspected as a determinant of the difference in the incidence of asthma in women and men after puberty.

Effect of Sex Difference on the Thickness of the Bronchial Epithelial Layer

Before the treatment of asthma modeling, there were differences in the thickness of the bronchial epithelial layer between male mice and female mice. However, this difference was not statistically significant. In female mice, the thickness of the bronchial epithelial layer was $18.9623 \pm 2.23 \mu\text{m}$, whereas, in male mice, the thickness was $15.2190 \pm 4.25 \mu\text{m}$. Modeling asthma with ovalbumin exposure in mice was proven to have caused changes in the thickness of the bronchial epithelial layer in both male and female mice, but these were also not statistically significant (respectively $p = 0.370$ and $p = 0.152$). This can be caused by the short period of ovalbumin exposure in the animal asthma modeling procedure chosen by the researcher.

The procedure of modeling asthma in experimental animals that the researchers used included a brief treatment with a total of 25 days and ovalbumin exposure for only three days, in contrast to the asthma modeling conducted by Li *et al.* (2013) with a total treatment for 52 and 43 days and with ovalbumin exposure for 14 days, or conducted by Yang (2005) with a total treatment for 52 days and ovalbumin exposure for six days, or by Mabalirajan *et al.* (2008) with a total

treatment of 27 days and ovalbumin exposure for seven days (Yang, 2005; Mabalirajan, *et al.*, 2008; Li, *et al.*, 2013). The relatively short exposure time in this study was able to cause an increase in the thickness of the epithelial layer of mice in both male and female asthma models but did not reach the statistical significance limit.

Meanwhile, there was no difference in the thickness of the bronchial epithelial layer between male and female mice in both the control mice group ($p = 0.085$) and the asthma model mice group ($p = 0.303$). This is in line with Blacquièrè *et al.*'s research, which states that sex difference (male or female) does not cause structural differences in the airways of experimental animals. However, significant differences are found in several other variables, such as eosinophil counts and cytokine levels (Blacquièrè, *et al.*, 2010).

Effect of Sex Difference on the Thickness of the Bronchial Smooth Muscle Layer

Before the treatment of asthma modeling, there were differences in the thickness of the bronchial epithelial layer between male mice and female mice. Supposedly, the difference is also not statistically significant, but this research showed significant results. In female mice, the thickness of the bronchial smooth muscle layer was $10.1379 \pm 1.75 \mu\text{m}$, whereas, in male mice, the thickness was $7.508 \pm 1.85 \mu\text{m}$. Modeling asthma with ovalbumin exposure in mice has been shown to cause changes in the thickness of the bronchial smooth muscle layer in both male and female mice. This was not statistically significant in female mice but was statistically significant in male mice ($p = 0.277$ and $p = 0.015$, respectively). This can be caused due to the reasons previously explained.

Meanwhile, the difference in thickness of the smooth muscle layer between male and female mice in the control mice group ($p = 0.030$ *), but not in the asthma model mice group ($p = 0.602$). This is in line with Blacquièrè *et al.*'s research, which states that sex difference (male or female) does not cause structural differences in the airways of experimental animals. However, significant differences are found in several other variables, such as eosinophil counts and cytokine levels (Blacquièrè, *et al.*, 2010).

Effect of Sex Difference on the Nasal Cavity's Goblet Cells Number

Before the treatment of asthma modeling, there had been differences in the number of nasal cavity's goblet cells between male mice and female mice. However, this difference was not statistically significant. In female mice, the number of nasal cavity's goblet cells (per 200 μm) was 2.1667 ± 1.59 , while in male mice, the number was 1.9583 ± 1.44 . Modeling asthma with ovalbumin exposure in mice was proven to have caused changes in the number of nasal cavity's goblet cells in both male and female mice, it was also statistically significant (respectively $p = 0.006$ * and $p = 0.002$ *). This is in line with research by Wang *et al.* (2008), and Zhang *et al.* (2019) related to synovial inflammation with methods similar to this study (using ovalbumin as exposure material) in which both images of goblet cell hyperplasia are obtained with a value of significant (Wang, *et al.*, 2008; Zhang, *et al.*, 2019).

Then, the exciting thing that can be observed is that in animal asthma modeling, histological structure changes (in this case is a significant difference in the number of goblet cells) are seen in the nasal cavity. The researchers' findings that the modeling of experimental animal asthma also caused a significant increase in the number of nasal cavity's goblet cells, which could be viewed as findings that support the theory of United Airway Disease. From an anatomical aspect, the nasal cavity is located in the upper airway while the bronchi are in the lower airway. Although both have anatomical and histological similarities such as the epithelium, lamina propria, and goblet cells, they are both very far apart. In research using animal models of this asthma, an inflammatory model was formed in the nasal cavity. This is in accordance with the theory of United Airway Disease or One Way, one disease which states that the airway is a unity, and the inflammatory process along it has a very similar etiopathogenic mechanism. Epidemiologically, clinically, and pathophysiologically, there is a strong relationship between airway diseases, especially asthma and rhinosinusitis (Marseglia, *et al.*, 2011; Licari, *et al.*, 2014; Giavina-Bianchi, *et al.*, 2016).

Meanwhile, there were no differences in the number of goblet cells between male and female mice in the control mice group ($p =$

0.816) and the asthma model group ($p = 0.140$). This suggests that the sex (male or female) does not cause structural differences in the airways of experimental animals (in this case, the number of goblet cells in the nasal cavity).

Effect of Sex Difference on the Thickness of the Nasal Cavity's Mucosal Layer

Before the treatment of asthma modeling, there were differences in the thickness of the mucous layer of nasal cavity between male mice and female mice. However, this difference was not statistically significant. In female mice, the thickness of the nasal cavity mucosa was $26.5022 \pm 1.68 \mu\text{m}$, whereas, in male mice, the thickness of the mucosa was $27.1473 \pm 3.55 \mu\text{m}$. Modeling asthma with ovalbumin exposure in mice has been shown to cause an increase in the thickness of the mucous layer of nasal cavity in both male and female mice, it is also statistically significant ($0.005 *$ and $0.003 *$ respectively). This is in line with Mendiola *et al.*'s (2016) study related to sino-nasal inflammation in ovalbumin-exposed mice, and although the method used is slightly different. However, this study is also in line with research by Kim *et al.* (2017) where the nasal cavity epithelial hyperplasia (which of course also affects the mucosa) in mice with asthma models exposed to proteases and ovalbumin (Mendiola, *et al.*, 2016; Kim, *et al.*, 2017).

Then, an exciting thing that can be observed is that in animal asthma modeling, changes in histological structure (in this case, the thickness of the mucous layer of nasal cavity) are seen in the nasal cavity. These results support the theory of the United States Airway Disease with the same explanation as previously reviewed.

Meanwhile, there were no differences in the number of goblet cells between male and female mice in the control mice group ($p = 0.699$) and the asthma model group ($p = 0.548$). This suggests that the sex (male or female) does not cause structural differences in the airways of experimental animals (in this case, the thickness of the mucous layer in the nasal cavity).

Conclusion

Experimental studies using mice (*Mus musculus*) which were exposed to ovalbumin as asthma models using 24 mice in four groups

(male control, female control, male treatment, and female treatment) as well as four variables (thickness of bronchial epithelium, bronchial smooth muscle thickness, thickness of bronchial smooth muscle, thickness of male nasal cavity's mucosa, and the number of nasal cavity's goblet cells) indicates that:

1. Sex difference (male or female) does not cause differences in the thickness of the epithelium and smooth muscle in the bronchus of mice (*Mus musculus*) asthma models, which is exposed to ovalbumin.
2. Sex difference (male or female) does not cause a significant difference in the number of goblet cells and thickness of mucosal in the nasal cavity of mice (*Mus musculus*) asthma models, which is exposed to ovalbumin.
3. Animal asthma modeling with ovalbumin exposure in this study proved to be able to cause an increase in the thickness of the bronchial mucous layer, thickness of the bronchial smooth muscle layer, the number of nasal cavity's goblet cells, and the thickness of the nasal cavity's mucous layer, both in male mice (*Mus musculus*) female, but sex difference (male or female) does not cause significant differences in the microscopic structure of the bronchi and the nasal cavity. Thus, it is suspected that other factors are more dominant (other than sex factors) that can explain the difference in the incidence of asthma in women and men, which still requires further research.

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