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計畫名稱：	建立以空氣細懸浮微粒 PM2.5 誘發過敏性結膜炎之小鼠模式

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Establishment of murine model of allergic conjunctivitis by particulate matter 2.5 (PM2.5)

Abstract

Many animal experiments have considered PM2.5 as an allergen and are suggested to be associated with allergic respiratory diseases or other inflammatory disease. However, whether PM2.5 causes eye allergy has not been tested. In this study, we determined the concentration of PM2.5 solution according to the air pollution standards set by environmental protection administration executive yuan, R.O.C (Taiwan) and directly stimulated by dropping to the eyes. We investigate whether different concentrations of PM2.5 cause allergic reactions and its pathological mechanism. In the clinical symptoms assessment, we found eyelid edema, tearing, and scratching with increased concentration. In addition, PM2.5 caused to structural abnormalities of conjunctival epithelium, goblet cells density decrease, and tendency for mast cells infiltration in conjunctiva. There are also structural abnormalities and keratinization in meibomian. The results demonstrate that PM2.5 indeed to induce allergic conjunctivitis.

Introduction

Particulate matter (PM), also known as atmospheric aerosols, including products from nature to artificial products such as pollen, dust, smoke, and industrial exhaust. PM2.5, whose diameter is less than or equal to 2.5 um, is more easily invade the human body and stay for longer periods of time. The harm to the body caused by long-term exposure is also more serious. These air pollution sources are the main cause of allergic diseases. Therefore, WHO offered Air Quality Guidelines (WHO, 2005) in 2005 to limit the concentration and various countries started to set up relevant standards. Many studies have pointed out that PM2.5 causes inflammation in the human body, including allergic respiratory diseases caused by nasal mucosa, such as asthma (Stephen H Gavett et al., 2003). It also causes damage to nasal mucosa and lung (Miao He et al., 2017, Zhiqiang Guo et al., 2017). In addition, studies also indicated that PM2.5 has an effect on vascular inflammation (Shengguang Chen et al., 2017) as well as neuronal inflammation and impairment of spatial memory (Tingting Ku et al., 2017).

However, allergic conjunctivitis is rarely mentioned as being associated with PM2.5. Allergic conjunctivitis, the major etiology is caused by the external environment of allergens, and its pathways divided into two: direct contact with the eye or indirectly through the nasal mucosa (Pelikan Z.2010). Its clinical symptoms including tearing, conjunctival swelling, edema, and eyelid edema (Takao Nakamura et al., 2003,

Jianping Chen et al., 2016, Mengyi Conga et al., 2017).In the process of inflammation, goblet cell, eosinophil, mast cell, and cytokines are involved (Daniel R. Saban et al., 2013, Hun Lee et al., 2017).Experimental allergic conjunctivitis models already have been development of many patterns by sensitization. The most common is intraperitoneal injection ovalbumin and therefore stimulate to eyes (Jian et al., 2016, Samuel Abokyi et al., 2014). Moreover, many studies have been conducted to determine the effect of ozone (Hun Lee et al., 2017), pollen (Claudia Córdova et al., 2014) ,and house dust mite (Young Ji Lee et al., 2016) on allergic conjunctivitis. Although other studies have pointed out that the concentration of PM2.5 is related to the number of people visiting hospital for allergic conjunctivitis (Mimura T et al., 2014). However, the mechanism by which PM2.5 causes allergic conjunctivitis remains unknown.

Here, we set up the concentration based on the PM2.5 sub-indicator of air pollution standard establish by environmental protection administration executive yuan, R.O.C (Taiwan) and evaluate whether PM2.5 induces allergic conjunctivitis. Assessment of allergic conjunctivitis with clinical symptoms, including eyelid edema, tearing, and scratching. In addition, the tear ferning was used to evaluate the quality of tears and the degree of changes in composition. It is also to explore whether PM2.5 effects on the cornea. Histology used to observe the cellular responses associated with inflammation and changes of structure such as goblet cells, mast cells in conjunctival epithelium and meibomian.

Materials and methods

PM2.5 reagent preparation

PM2.5 is provided by the laboratory of Associate Professor Lai Quanyu, Department of Occupational Safety and Health, Sun Yat-sen University. The sample was taken using PM-10 high-volume sampler of model TE-6070V PM10 sampler. The sampling area was based in Taichung, Taiwan. According to the pollution sub-indicator of interval concentration, we extracted the stock solution to the desired concentration and set up the corresponding concentration to 50 μg/m^3, 100 μg/m^3, and 200 μg/ m^3 (Figure 1).

污染物濃度與污染副指標值對照表

空氣品質指標(AQI)							
AQI指標	O_3 (ppm) 8小時平均值	O_3 (ppm) 小時平均值 (1)	$PM_{2.5}$ ($\mu g/m^3$) 24小時平均值	PM_{10} ($\mu g/m^3$) 24小時平均值	CO (ppm) 8小時平均值	SO_2 (ppb) 小時平均值	NO_2 (ppb) 小時平均值
良好 0～50	0.000 - 0.054	-	0.0 - 15.4	0 - 54	0 - 4.4	0 - 35	0 - 53
普通 51～100	0.055 - 0.070	-	15.5 - 35.4	55 - 125	4.5 - 9.4	36 - 75	54 - 100
對敏感族群不健康 101～150	0.071 - 0.085	0.125 - 0.164	35.5 - 54.4	126 - 254	9.5 - 12.4	76 - 185	101 - 360
對所有族群不健康 151～200	0.086 - 0.105	0.165 - 0.204	54.5 - 150.4	255 - 354	12.5 - 15.4	186 - 304 (3)	361 - 649
非常不健康 201～300	0.106 - 0.200	0.205 - 0.404	150.5 - 250.4	355 - 424	15.5 - 30.4	305 - 604 (3)	650 - 1249
危害 301～400	(2)	0.405 - 0.504	250.5 - 350.4	425 - 504	30.5 - 40.4	605 - 804 (3)	1250 - 1649
危害 401～500	(2)	0.505 - 0.604	350.5 - 500.4	505 - 604	40.5 - 50.4	805 - 1004 (3)	1650 - 2049

Figure 1.: the standard of air pollution including many sub-indicator of corresponding concentration,set by environmental protection administration executive yuan,R.O.C(Taiwan)

Animal experiment

Fifty female ICR mice (Lasco, Taiwan), aged 6-8 weeks, were randomly divided into five groups: (1) blank group, eyes were stimulated with 0.9% saline, (2) 50 μg/day group, daily concentration of each eye contact up to 50 μg, (3) 100 μg/day group, daily concentration of each eye contact up to 100 μg, (4) 200 μg/day group, daily concentration of each eye contact up to 200 μg, and (5) 200 μg/day + AT group, in order to observe whether artificial tears have a soothing effect on allergic conjunctivitis, daily exposure to a concentration of 200 μg and therefore treated with artificial tears after stimulation. The tests of day 0 were performed before stimulation to construct baseline. On the next day, the mice were instilled into each eye with 5.2 μl of the corresponding concentration of reagent three times a day, routinely at 9:00 a.m., 1: 00 pm, and 5:00 pm. The process was up to 19 days. On day 9 and day 18, clinical symptoms were evaluated between 15-30 minutes after exposed to PM2.5. At day 19, the mice were anesthetized with avastin by

intraperitoneal injection for assessment of tear ferning and corneal surface photography. The mice then sacrificed to collect blood, eyes, lacrimal gland, and meibomian to further analysis.

Evaluation of clinical symptoms and corneal assessments

Clinical manifestations of allergic conjunctivitis include increased tear volume, eye edema, swelling of the conjunctiva, and itching (Takao Nakamura et al., 2003, Jianping Chen et al., 2016, Mengyi Conga et al., 2017) Here, we test tear volume with 1-mm width strip cut off from PH paper, and tear break-up time (TBUT), which is that both cornea from each mice were stained with 1% fluorescein for observing times of tear film rupture to evaluate the amount and qualities of tears (Cho P. et al., 2004, Bo-Yie Chen et al., 2013) . In addition, eyelid edema was evaluated by photograph. In behavioral assessment, we referenced the way used in literature and made modifications. The mice were isolated from each other and in the cage placed individually for at least an hour to adapt to the environment, their scratch performance was defined as each hind limbs to capture the eye, and after the hind legs dropped to the ground which was to be regarded as the end of a round, and using video recording to record the number of scratching round in 30 seconds (Steven G. Shimada and Robert H. LaMotte, 2008). Furthermore, to evaluate whether the behavior of scratching hurts the surface of cornea, images of surfaces were taken with a stereoscopic zoom microscope equipped with ring illuminator (SMZ 1500; Nikon) to assess smoothness, topo, opacity and staining by lissamine green. (Yie Chen et al., 2013)

Tear ferning

Tear ferning is clinically used to assess tear quality in patients with dry eye and its crystalline form changes with the patient's tear composition and external environment (Masmali AM et al., 2014, Mimura T et al. al., 2014, Leonidas Traipe-Castro et al., 2014). Here we use 2 µl of 0.9% saline to repeatedly wash each eye about 6-7 times and drop the washed liquid directly onto the glass slide. In the process of air drying, maintained a constant environment temperature, humidity, and avoided dust.

Histology evaluation

After using carbon dioxide to sacrifice mice, the eyes including eyelid and lacrimal gland were isolated. After tissue embedding, the 5 µm tissue sections were processed for hematoxylin–eosin (HE) stain to observe the structure changes of conjunctival epithelium. Periodic acid Schiff (PAS) stain used to evaluate the goblet

cells. Moreover, Giemsa stain was performed to assess the infiltrated number of eosinophils and mast cells.

Statistical analysis

All data were obtained from triple repeats and are presented as the mean 6 standard error of the means (SEM) and compared among groups by using nonparametric statistics. The program SPSS (IBM Software, version 22) were used to analyze the data.

Results

Effects of PM2.5 on clinical symptoms in allergic conjunctivitis model

To investigate whether different concentrations of PM2.5 successfully induced allergic conjunctiva in this study, we evaluated the clinical symptoms including eyelid edema, tearing, and behavior. In the tear volume test, the result showed that at the day 9 and day 18, there was a significant increase in 200 μg/day group and 200 μg/day + AT group compared to blank group(Figure 2). We suggested that as the concentration of PM2.5 increased, tear volume also increased. We next analyzed the TBUT that reflects tear quality. Interestingly, the tear film break-up time of 100 μg/day group, 200 μg/day group and 200 μg/day + AT group were found to be significantly increased on day 18 (Figure 3). In addition, we evaluated the eyelid edema by photography at day 0 (baseline), day 9, and day 18 (Figure 4A). The results showed that the extent of eyelid edema was more pronounced with increasing concentrations compared to the baseline and blank group (Figure 4B). In assessment of behavior, we calculated the number of round of scratching by video recording (Figure 5B). After analyze, we found no significant difference between all the groups (Figure 5A). From the overall clinical characterization assessment, it was found that as the concentration of PM2.5 was higher, the symptoms of mice showed more obvious. Moreover, the 200 μg/day + AT group also showed that the artificial tear relieved the symptoms of allergic conjunctivitis caused by PM2.5 are slightly.

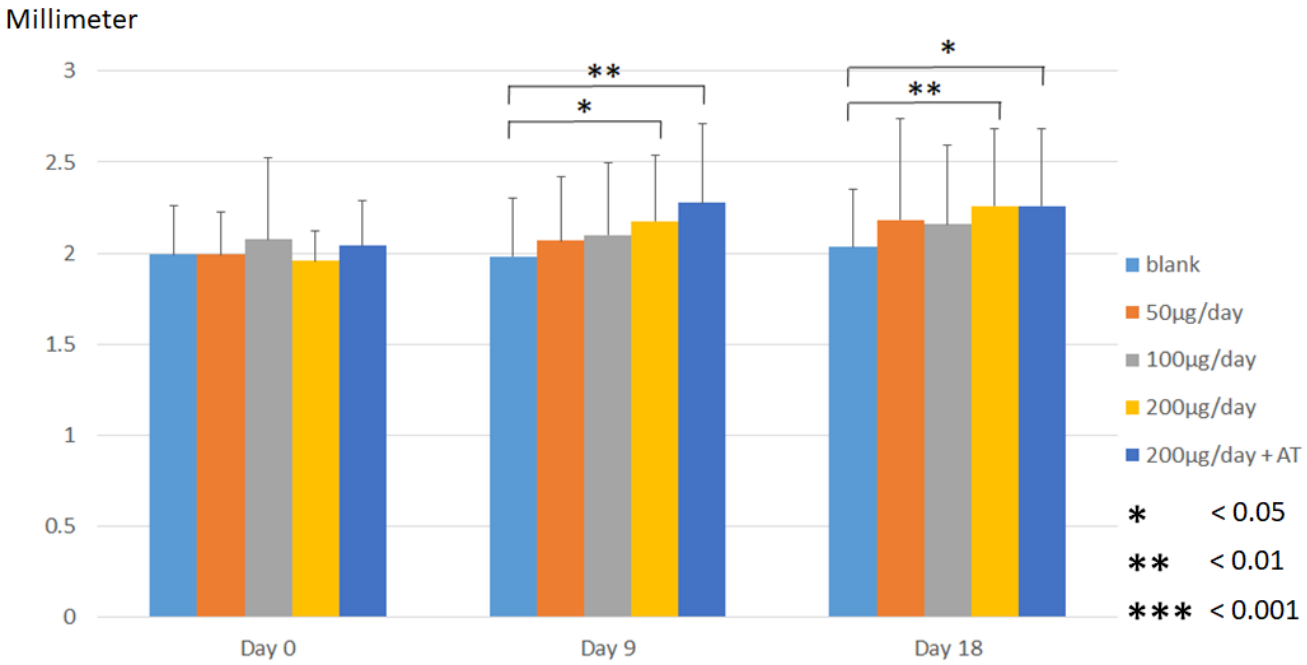

Figure 2.:Aqueous tear production (in millimeter) assessed with a 1-mm-width strip cut off from a pH test paper at day 0,9 and 18 of the experiment .

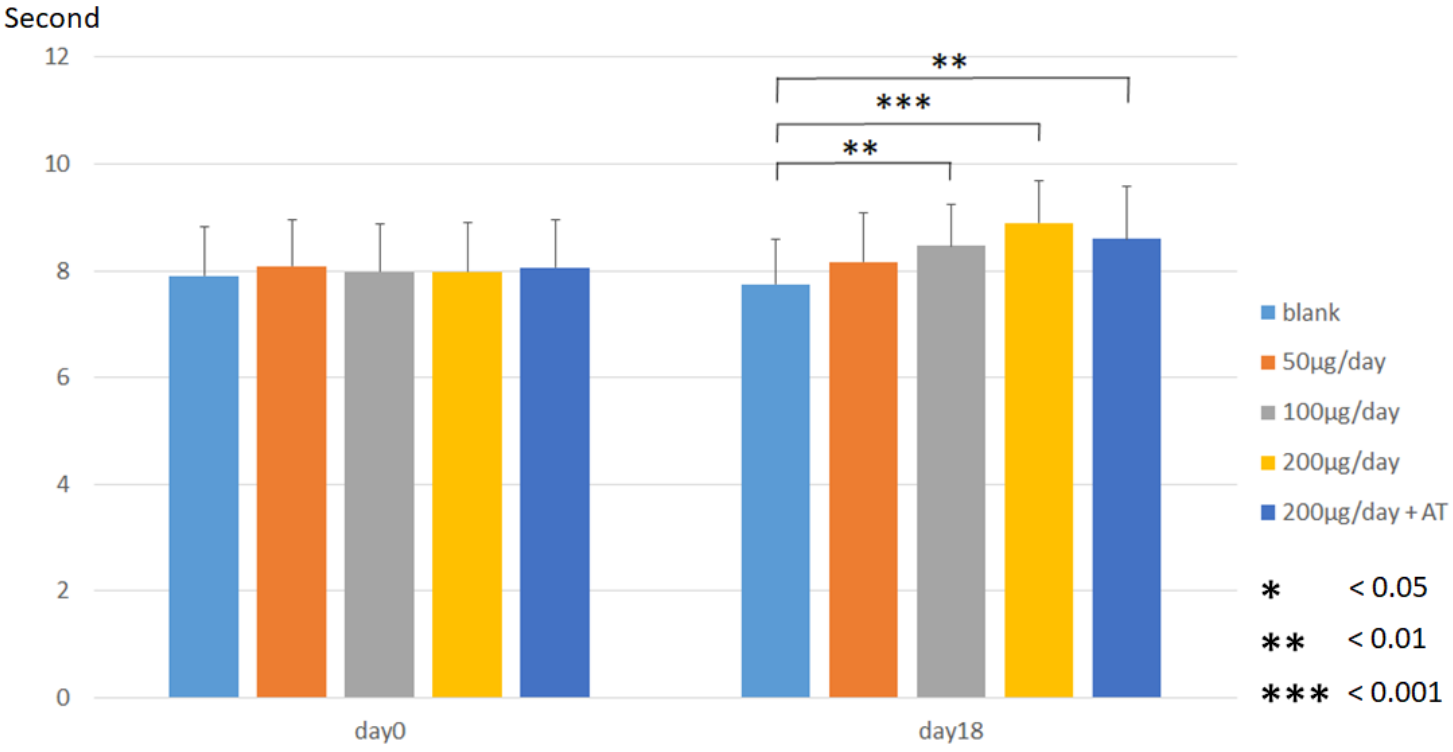

Figure 3.:Tear film breakup time (in seconds) assessed after fluorescein staining and recording at day 0 and 18 of the experiment.

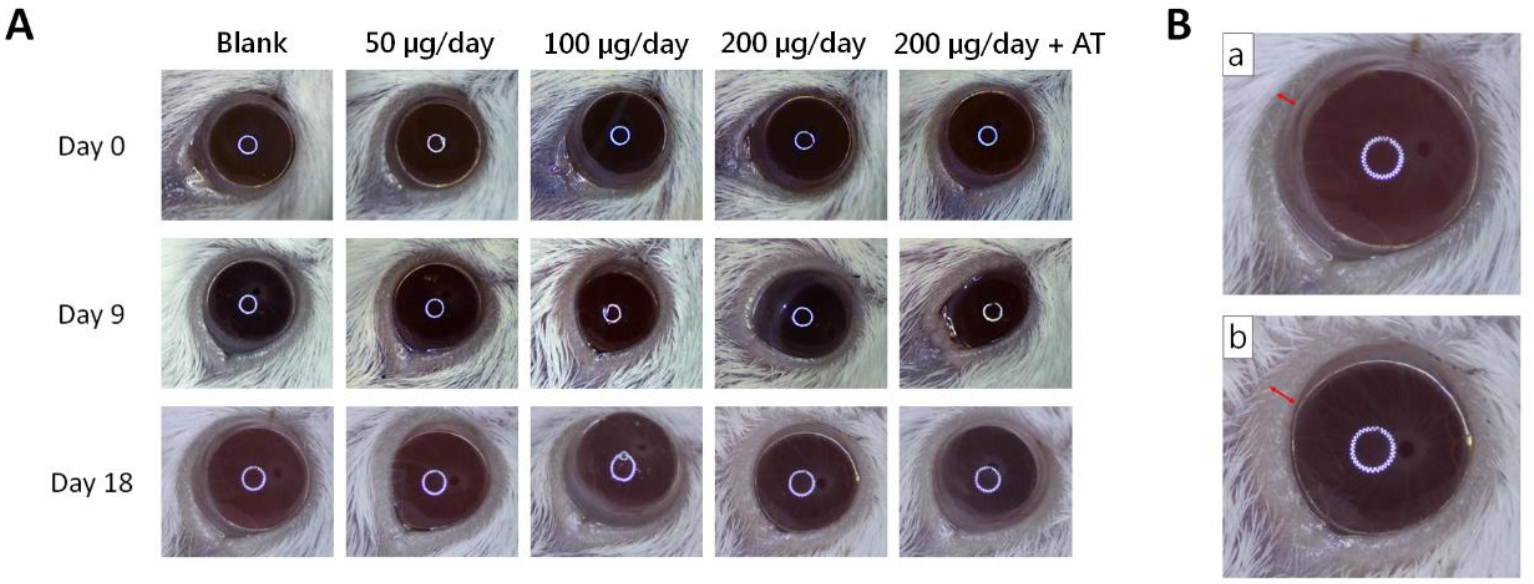

Figure 4.:(A)Eye lid edema of clinical symptoms assessed with photography at day 0,9 and 18 of the experiment. (B)At day 18,eye lids of 200 μg/day group (the figure b) is more swollen as compared to blank group (the figure a).

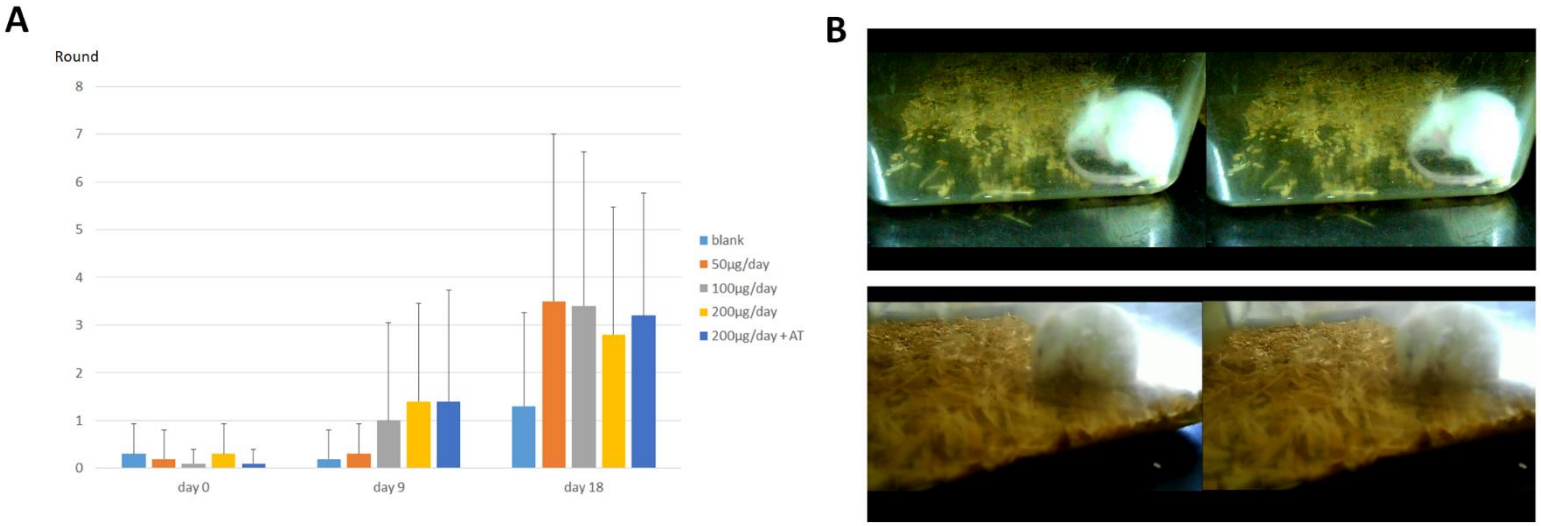

Figure 5.: (A)Scratching times (in rounds) assessed with vedio and recording at day 0,9 and 18 of the experiment. (B)We use video to record and evaluate the scratching behavior of mouse.

Assessment of PM2.5 on corneal surface photography

The cornea damages were assessed by opacity, smoothness, topography, and lissamine green staining. The results of the day 19 showed that except for opacity and smoothness, there were a certain degree changes in topography and lissamine green staining as the concentration increased, which is significant in 200μg/day group (Figure 6). In addition, the 200μg/day + AT group in topography and lissamine green staining showed a slight reduction compared with 200μg/day group. It is suggest that the behavior of scratching caused by PM2.5 may injure the cornea without prompt treatment. Furthermore, although artificial tears have limited

alleviation of clinical symptoms, it is possible to avoid the cornea being damaged.

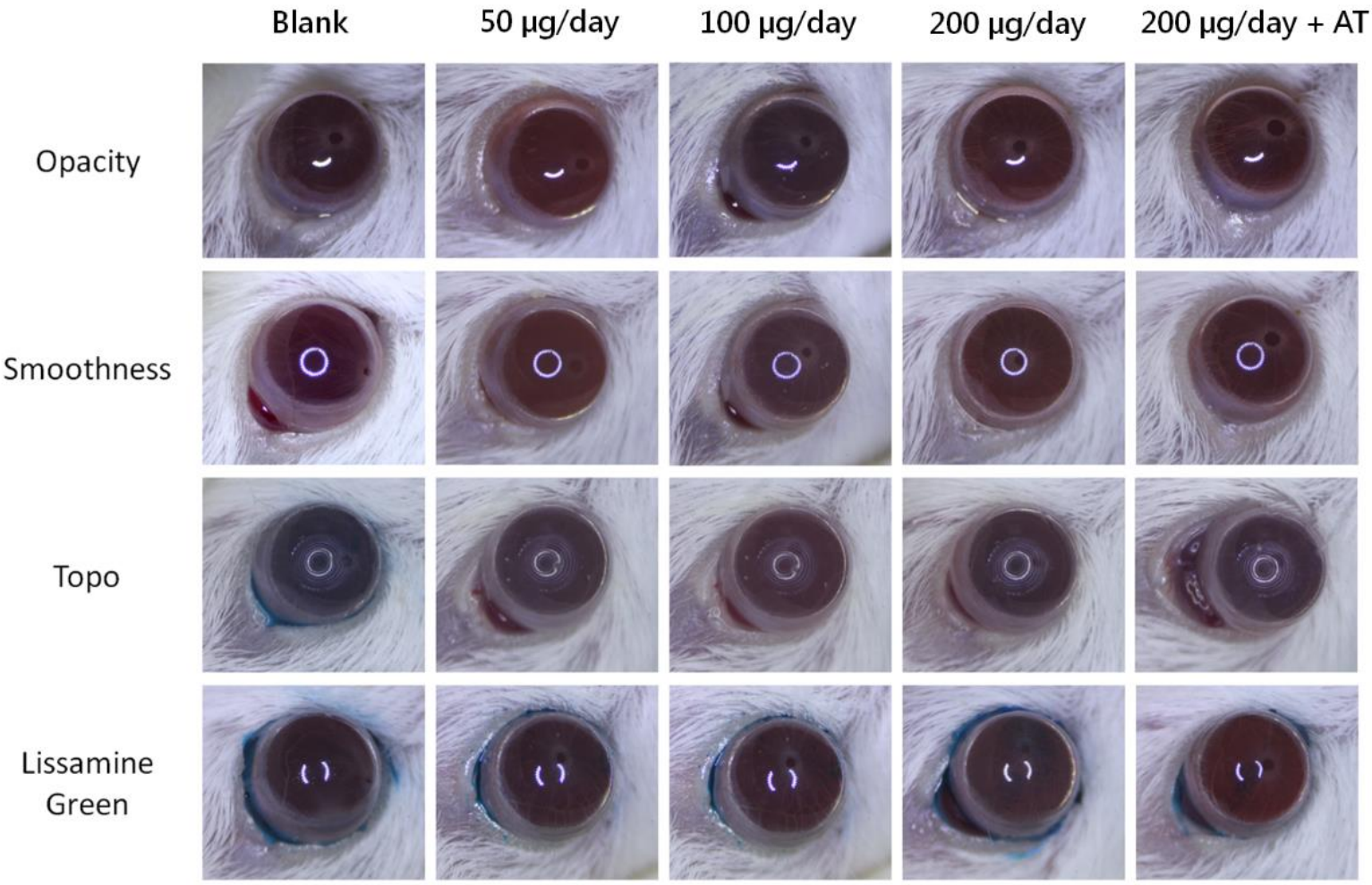

Figure 6.: Ocular surface assessments for corneal smoothness, opacity, topography, and extent of lissamine green staining at day 19 of the experiment

The pattern of tear ferning

Tear ferning already has many grading patterns (Rolando M.,1984, Vaikoussis E et al.,1994, Norn M.,1987, Ali M Masmali et al.,2014).Through evaluating the dimension of ferns and the spacing between the branches in the ferns to assess the type of ferning and its severity. With the pathological changes become serious, the ferns become smaller and incompletely formed with rare or no branching and the gaps become larger and wider, even the ferning phenomenon will disappear (Masmali AM et al., 2014). Here, the results show that as the concentration increases, the less fernlike crystals formed, which means more severe deterioration of the tear (Figure 7). It indicates that PM2.5 alters the tear composition, and it probably affects the symptom of tearing to deteriorate allergy.

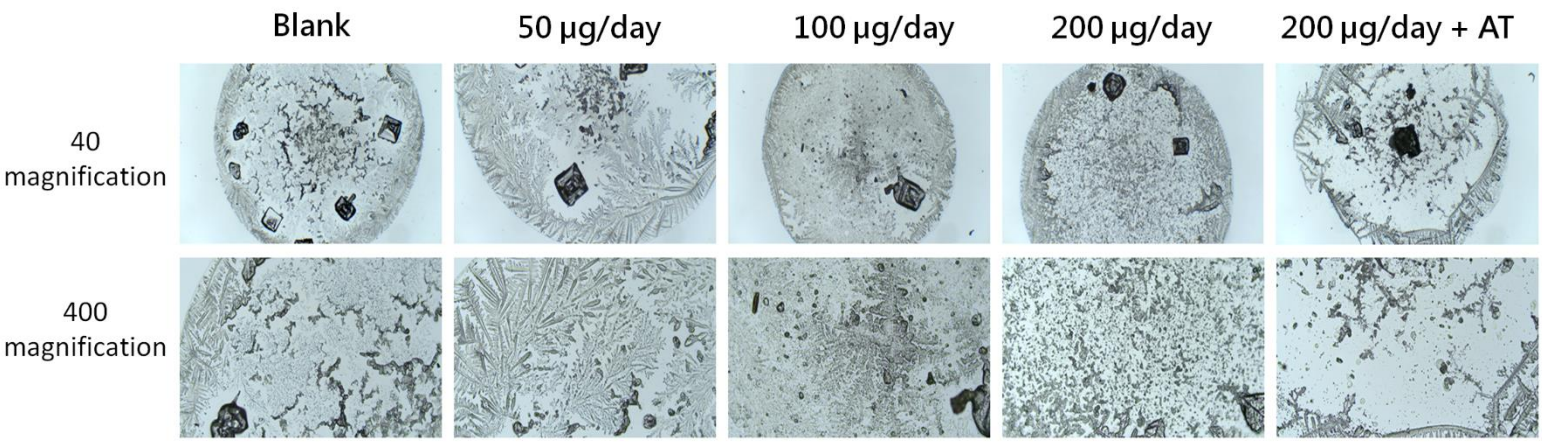

Figure 7.: Tear ferning assessed with 40 magnification and 400 magnification at day 19 of the experiment

Analysis of histology

Studies have shown that the goblet cells distributed in the conjunctiva may be affected by cytokines, such as IL-13, TNF-a, and thus affect its proliferation, apoptosis, and its mucin secretion (Darlene A. et al., 2014). .In addition, mast cell would be found in conjunctiva and epithelium in ocular allergy, and be a mediator of eosinophils (Dai Miyazaki et al., 2008). Also studies have indicated that the number of goblet cells will decline after six hours of final allergen stimulation and recover after 48 hours (Kathleen S. et al., 2001). We have found that the conjunctiva structure becomes abnormal as the concentration of PM2.5 increases (Figure 8).There is an abnormal decrease in the number of goblet cells can be observed through PAS stain. In Giemsa stain, it seems that mast cells increase as the concentration increases. However, Giesma stain can also be used to evaluate eosinophils, but it still needs further analysis in the future to confirm whether eosinophils exist in epithelium.

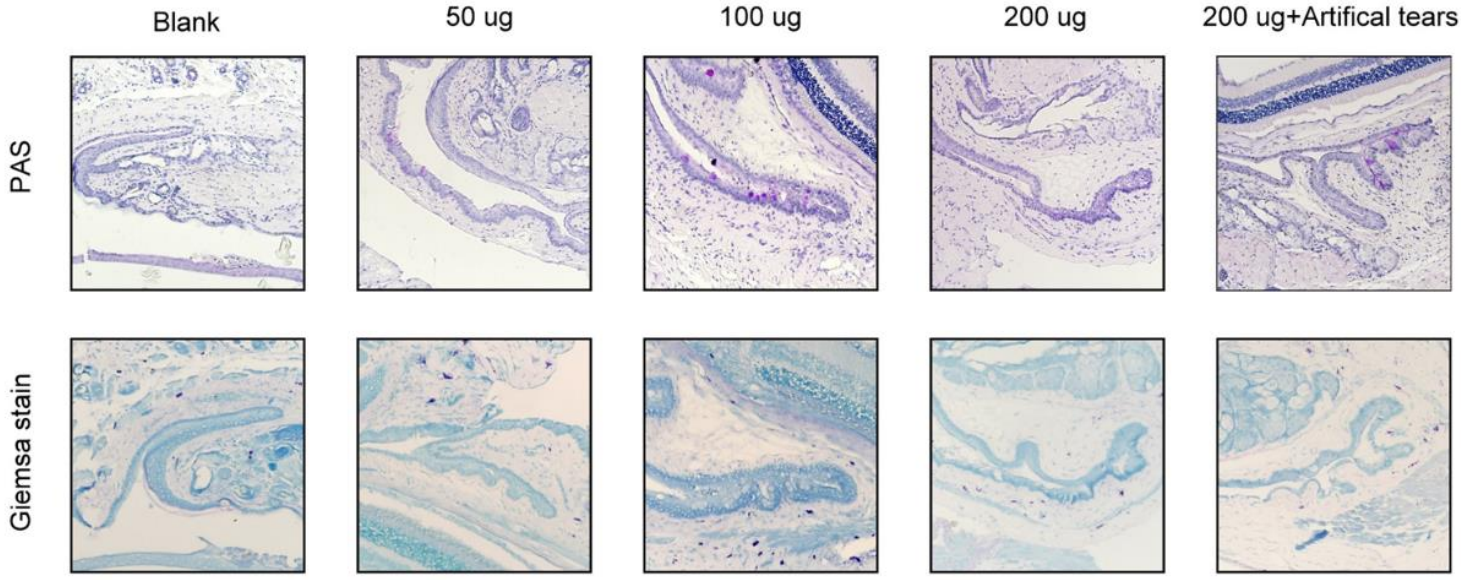

Figure 8.:Histological analysis of conjunctiva and tissue of epithelium at day 19 of the experiment after PAS staining and Geimsa staining.

In analysis of meibomian, some studies indicated that meibomian gland dysfunction would result in alteration of the tear film, clinically apparent inflammation, and ocular surface disease(Hideki Miyake et al.,2017).Furthermore, the meibomian glands exhibited a thickening, hyperkeratinization of ductal epithelium, and atrophy of the acinar component was also observed in the glands(Hideki Miyake et al.,2017, Hideki Miyake et al.,2016).Interestingly, our studies were found that when the concentration increased, the meibomian also had a tendency of keratinization and ductal occlusion (Figure 9). Furthermore, we also used Giemsa stain to determine whether mast cells exist in the meibomian gland.

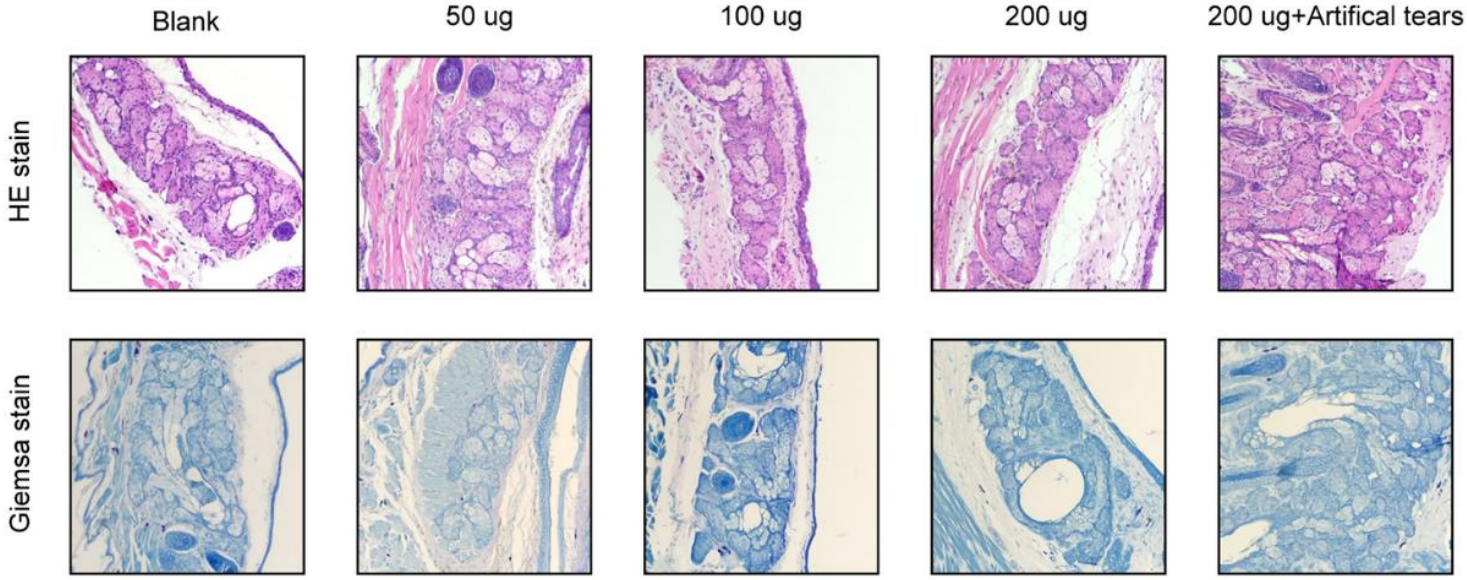

Figure 9.:Histological analysis of Meibomian at day 19 of the experiment after Hematoxylin-Eosin staining and Geimsa staining.

Discussion

At present, many studies know about allergic conjunctivitis caused by air pollutants such as pollen or ozone. However, the mechanism of allergic conjunctivitis induced by PM2.5 is still incomplete. However, based on this model, it can be extended and develop more application-oriented in the future.

This study differs from the general experimental allergic conjunctivitis in that we did not use intraperitoneal injection of ovalbumin or PM2.5 to induce an allergic reaction. Modern people pay attention to the air quality and carry masks, and thus reduce the amount of allergens inhaled. However, the eye still has a great chance of exposure to allergens and is also more likely to cause chronic and long-term minor

eye diseases, which will still cause problems in life. Therefore, this study is more realistic in nature. In addition, there are studies perform the way of stimulation is using the gas release form to sensitize the eye (Hun Lee et al., 2017), which is the way the model can be modified in the future.

In the clinical context, as well as corneal evaluation, there is literature that uses conjunctival fluorescein staining to assess conjunctival damages(Hun Lee et al., 2017).We only used lissamine Green staining to observe whether the cornea was also affected (Korb DR et al., 2008, Eom Y et al., 2015),which is also a part of the model that can be tried in the future in order to observe the effect of PM2.5 on the conjunctiva damage, and extended to use a variety of ways to observe the conjunctival changes.

This study also explored the scratching behavior of mice. One study has shown that mice with severe allergic conjunctivitis may not scratch their legs but may experience decreased activity (Young Ji Lee et al., 2016). Interestingly, the 200μg/day group had this phenomenon (Figure 8) which is that the future can be used as one of the assessments. However, it has been reported in literature that scratching behavior in mice can easily be affected by extrinsic sounds or environmental familiarity (Steven G. Shimada and Robert H. LaMotte, 2008), even with conditioned place aversion (Di Mu, Yan-Gang Sun, 2017).These factors are also limitations of this study and is part of the future to observe the behavior of mice through this model.

The results of tear ferning showed changes in tear quality as the concentration of PM2.5 increased, which may also be responsible for the data of tear volume and TBUT. In allergic conjunctivitis, the compositions of tear include cytokines, such as specific IgE (Tatsuya Mimura et al., 2013), eotaxin (Simone Eperon et al., 2014), Interleukin and leukotrienes (Zdenek Pelikan et al., 2013),and it divided into watery layers, mucin layers and lipid layers.Allergic conjunctivitis has been reported to cause in particular the instability of mucin layers and lipid layers (Kaijun Li et al., 2010), and in recent years there has been study showing that the pattern of tear ferning in keratoconjunctivitis sicca(David Williams and Heather Hewitt, 2017).Interestingly, the clinical symptoms of keratoconjunctivitis differ from which of allergic conjunctivitis, but the results of both tear ferning are very similar.If in the future ,we can get enough mouse tears for analysis, we can try to analyze the changes in composition of tear,and further identify what kind of protein or cytokine lead to tear ferning crystallization anomalies to expand the model. In addition, in the pathological section,abnormality and reduction of goblet cells can be found, which can also be proved that the mucin secreted by the goblet cells is affected, and the presence of inflammation-associated mast cells are also found. Furthermore, with the PM2.5 concentration increase, the meibomian structure produces abnormalities. These

factors will lead to altering the tear film and inflammation of eyes It can be known that prolonged contact with allergens causes the underlying structures of the eye to be fundamentally damaged, leading to long-term chronic effects.

This study pioneered a new experimental allergic conjunctivitis model path, although there are still some limitations and can be modified part, but it is for future development and extension of the foundation. The clinical literature pointed out that PM2.5 may one of the reasons is related to the number of people visiting the hospital for allergic conjunctivitis (Mimura T et al., 2014),furthermore, the excessive number of people who seek medical care can also lead to the burden of related medical services and resources. It can even cause long-term chronic diseases. So it is why this experimental model is important. Through using this research as a foundation to develop the PM2.5 damage and effects on the eye, and through this model to explore possible mitigation, corresponding strategies, or even other research-oriented expansion, it will enable people to more focus on the external environment for the impact of pollutants on allergic conjunctivitis, and helps to ease the medical burden.

Figure 10.: In the evaluation of scratching behavior, it was found that there was a decrease in motility of 200 μg / day in the group with closed eyes, possibly reflecting the discomfort caused by PM2.5.

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