THE EFFECT OF AEROSOLISED-HONEY ON INFLAMMATION, REGENERATION AND REPAIR OF AIRWAY EPITHELIA IN RABBIT MODEL OF OVALBUMIN-INDUCED CHRONIC LUNG INJURY

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by

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LIST OF ABBREVIATIONS

AB-PAS	Alcian-blue periodic acid Schiff
AHR	Airway hyperresponsiveness
Alum	Aluminum hydroxide
BAL	Bronchoalveolar lavage
BV	Bronchial vessel
C	Cartilage
COPD	Chronic obstructive pulmonary disease
DM	Diabetes mellitus
ECM	Extracelullar matric
ELISA	Enzyme-linked immunosorbent assay
Eo	Eosinophil
Ep	Epithelial layer
GC	Goblet cell
GIT	Gastrointestinal tract
HDM	House dust mite
H&E	Haematoxylin and eosin
ICS	Inhaled corticosteroids
IFNG	Interferon gamma
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IHC	Immunohistochemistry
IL-4	Interleukin-4

IL-8	Interleukin-8
IL-5	Interleukin-5
IL-13	Interleukin-13
i.p.	Intraperitoneal
LABA	Long acting β-agonist
LAMA	Long acting muscarinic antagonist
M	Macrophage
MAPK	p38-mitogen-activated protein kinase
MUC	Mucin
NHSM III	Third National Health and Morbidity Survey
OVA	Ovalbumin
PBS	Phosphate buffer saline
Rb	Retinoblastoma
RBC	Red blood cell
RBM	Reticular basement membrane
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-beta
TH	Tualang honey
Th1	T-helper type 1
Th2	T-helper type 2
SABA	Short acting β-agonist
SAMA	Short acting muscarinic antagonist
SD	Standard deviation
SM	Smooth muscle

LIST OF SYMBOLS

Mg	Milligram
mL	Millilitre
Kg	Kilogram
mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitre
%	Percentage
°C	Degree Celsius
Mm	Millimetre
µm	Micrometre
µL	Micro litre
<	Less than
>	Greater than
®	Registered trademark
RPM	Revolutions per minute

KESAN PENGGUNAAN SEMBURAN MADU KE ATAS KERADANGAN, REGENERASI DAN BAIK PULIH EPITELIA SALURAN PERNAFASAN PADA MODEL ARNAB YANG MENGALAMI KECEDERAAN PEPARU KRONIK DIAKIBATKAN OLEH OVALBUMIN

ABSTRAK

Selama beberapa dekad, penyakit kecederaan peparu kronik telah mengakibatkan ribuan kematian di seluruh dunia. Penyakit ini terjadi apabila saluran pernafasan mengalami perubahan dari segi struktur dan komponen-komponen sel di sekelilingnya. Penyakit ini tidak boleh disembuhkan, namun begitu, pelbagai jenis rawatan telah tersedia untuk membantu mengurangkan gejala-gejala penyakit dan seterusnya menghalang penyakit ini daripada bertambah serius. Di peringkat klinikal, terapi berasaskan ubat-ubatan telah diperkenalkan untuk membantu mengurangkan gejala serangan penyakit ini. Walau bagaimanapun, usaha bagi mengenalpasti rawatan alternatif bagi penyakit ini berasaskan bahan-bahan semulajadi masih diteruskan dengan tujuan untuk mengurangkan kesan-kesan sampingan akibat penggunaan terapi ubat-ubatan. Objektif umum bagi kajian ini adalah untuk mengenalpasti kesan rawatan semburan madu ke atas pembaikpulihan dan regenerasi sel-sel epitelia saluran pernafasan pada model arnab yang mengalami kecederaan peparu kronik diakibatkan oleh ovalbumin. Model arnab telah diberikan kecederaan melalui suntikan ovalbumin, dan seterusnya rawatan semburan madu Tualang telah diberikan menggunakan penebula. Model rawatan telah dibahagikan kepada dua kumpulan iaitu untuk mengenalpasti kemampuan madu Tualang untuk bertindak sebagai agen penyelamat dengan cara memberi rawatan kelegaan segera atau sebagai

agen penghalang (untuk mengelakkan kecederaan berulang). Kajian ini telah dilakukan menggunakan dua kepekatan madu Tualang (*Koompassia Excelsa*) yang berbeza; 25% dan 50%. Kajian ini memfokuskan kesan histologi rawatan semburan madu terhadap tiga parameter kecederaan peparu kronik iaitu; (1) membaikpulih struktur saluran pernafasan, (2) mengurangkan bilangan dan/atau menyingkirkan sel-sel goblet, dan (3) mengurangkan kemasukan sel-sel keradangan ke dalam saluran pernafasan. Rawatan semburan madu ini (tanpa membezakan kepekatan madu yang digunakan dan mod rawatan) memberi keputusan yang memberansangkan apabila ianya mampu mengurangkan gejala-gejala penyakit ini dengan mengembalikan semula struktur saluran pernafasan, mengurangkan bilangan sel-sel goblet dan mengurangkan kemasukan sel-sel eosinofil ke dalam bahagian tisu peparu. Dapatan daripada kajian ini menunjukkan rawatan semburan madu boleh bertindak sama ada sebagai agen penyelamat dan juga agen penghalang; dan seterusnya mempunyai potensi untuk digunakan sebagai rawatan alternatif bagi penyakit kecederaan peparu kronik pada masa akan datang.

THE EFFECT OF AEROSOLISED-HONEY ON INFLAMMATION, REGENERATION AND REPAIR OF AIRWAY EPITHELIA IN RABBIT MODEL OF OVALBUMIN-INDUCED CHRONIC LUNG INJURY

ABSTRACT

For the past few decades, chronic lung injury has attributed to thousands of death worldwide. The disease occurred due to alteration in the structure as well as cellular components of the airways which later lead to airway remodelling and inflammation. This disease is not curable; however, treatments are available to reduce the disease symptoms and preventing the disease progression. At the clinical setting, conventional drug therapy is available to lessen disease exacerbations; however, there is still a need to find the alternative treatments that incorporated with natural resources to minimize the side effect of drug therapy. The general objective of this study was to study the effect of aerosolised-honey on airway epithelium repair and regeneration following ovalbumin-induced chronic lung injury in rabbit model. Injury was first introduced in the rabbit model by ovalbumin sensitization and treatment of aerosolised-Tualang honey was given to the animals using a nebuliser. Two models had been developed with the aim to investigate the potentiality of Tualang honey to serve as rescue agent by providing quick relief upon disease symptoms or as preventive agent (to prevent the progression of the disease). Research was carried out using two concentrations of Tualang (*Koompassia Excelsa*) honey; 25% and 50%. Current study was focussing on the histological effect of aerosolised-honey treatment towards three parameters of chronic lung injury; (1) restoration of airway structures, (2) improving and/or eliminating the goblet cell hyperplasia, and (3) reducing the inflammatory cells infiltration. Results between the

treatment groups, naive and control groups were statistically compared. Aerosolised-Tualang honey (regardless of any concentration and modes of action) was shown to be able to alleviate the disease symptoms by restoring the airway structures, eliminating the goblet cell hyperplasia and reducing the eosinophils infiltration. These findings provide the evidence that aerosolised-honey can act as both rescue and preventive agents, thus it could be useful to be applied as alternative treatment in managing the chronic lung injury patients in future.

CHAPTER 1

INTRODUCTION

1.1 Lung disease

Lung disease is disease affecting airways and other structures of the lung. It is the most common medical complication been reported worldwide and the disease can be divided into two categories; acute and chronic. Acute respiratory disease is described as the condition of abnormal lung function when exposed to certain stimuli and can be developed due to factors such as lung infection, inhalation of highly toxic gases, sepsis and others (Kamaruzaman et al., 2013).

Meanwhile, the development of chronic lung disease is often associated with smoke inhalation, genetic and occupational environment (Kamaruzaman et al., 2013). Chronic lung disease is estimated to affect hundreds of millions of people worldwide and examples of these non-communicable disease are asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, bronchitis, emphysema, lung cancer and lung fibrosis (World Health Organization, 2007). Disease outcomes are including airway inflammation, airway hyperresponsiveness (AHR) and mucus overproduction (Kamaruzaman et al., 2013).

1.2 Chronic lung injury – COPD

COPD is a major public health problem and listed as the major and fourth leading cause of morbidity and mortality in the United Kingdom and United States respectively (Rabe et al., 2007, Parmar and Raines, 2015). There are about 16

millions of people diagnosed with COPD and most commonly seen in elderly patients (Rabe et al., 2007).

COPD is characterised by poorly reversible airflow limitation with chronic inflammation in the lungs that occurred as a result from long terms exposure towards noxious particles and gases particularly cigarette smoke. The disease is progressive, often develop slowly and the symptoms worsen over time (Rabe et al., 2007).

The pathological changes of the disease occurred throughout the pulmonary zones including proximal cartilaginous airway, peripheral airway, lung parenchyma and pulmonary vasculature (Pauwels et al., 2001, MacNee, 2006). The inflammatory and airway remodelling increased with disease severity and persist even after smoking cessation (MacNee, 2006). As a result, these lead to the accumulation and infiltration of inflammatory cells such as macrophages, T lymphocytes (predominantly $CD8^+$) and neutrophils into airway regions. Soon, this contributes to the release of mediators by inflammatory cells such as interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), leukotriene B4 (LTB_4) and others, which later resulting in damaging of the lung structures and sustaining of neutrophilic inflammation (Pauwels et al., 2001, MacNee, 2006).

The pathophysiologies of COPD including mucus hypersecretion, cilliary dysfunction, airflow limitation, pulmonary hyperinflation, gas exchange abnormalities and pulmonary hypertension.

Mucus hypersecretion occurred due to squamous metaplasia, increased in the number of goblet cells and increased the size of bronchial submucosal glands in response to chronic irritation by noxious particles and gases. As a result, patients

with COPD will experience chronic productive cough and sputum production (MacNee, 2006).

Meanwhile, cilliary dysfunction occurred due to squamous metaplasia of epithelial cells. As the consequence, this results in abnormal mucocilliary escalator and patients will having difficulty in expectorating (MacNee, 2006).

Chronic inflammation leads to persistent cycles of airway wall repairing and later resulted in the structural remodelling of the airway wall. In addition, increasing collagen content and scar formation within the airway wall leads to airway lumen narrowing and thus producing fixed airway obstruction (Pauwels et al., 2001). This condition is worsening by the presence of inflammatory exudates in the small airway (less than 2 mm internal diameter) (MacNee, 2006).

In overall, patients of COPD will experience clinical symptoms like shortness of breath, chronic cough and sputum production (Pauwels et al., 2001).

1.3 Chronic lung injury - asthma

Asthma is the most common chronic inflammatory airway disease that affecting millions of people worldwide. In 2004, it has been reported that about 300 millions of people globally had asthma with 250,000 annual deaths attributed by the disease (Masoli et al., 2004, World Health Organization, 2007). In Malaysia, according to the Third National Health and Morbidity Survey (NHMS III) conducted in 2006, the prevalence rate of asthma among adults has been reported about 4.5% and the rate was estimated to be rising especially in children (Usha Devi et al., 2011). In a research focusing on the prevalence of childhood asthma in Selangor, Malaysia,

the prevalence rate of asthma among children was reported to be 24% (Surdi Roslan et al., 2011).

In a healthy individual, the process of inhaling and exhaling of air occurred freely without any obstruction. However, in individuals with asthma, the muscle surrounding the airway became constricted, thus led to difficulty in breathing. This was worsening by the inflammation of airway which contributed to breathlessness and chest tightness. Asthma is characterized as repetitive disease of the airway thus individuals with asthma had been diagnosed to have recurrent episodes of wheezing, breathlessness, chest tightness and nocturnal coughing (Kamaruzaman et al., 2014).

Usually this disease is treated by means of medication. Delay in seeking treatment for the disease may lead to serious asthma attack and fatality. For asthmatic individuals, it is important to manage the disease with proper management which including taking the medication and practicing the healthy lifestyle. Improper asthma management may hinder an individual from enjoying the life and increase their life burden. According to a global research known as Spring into Action, about 90% of asthmatic individuals is reported to have difficulty in participating in any physical activity and at least 70% of the respondents claimed asthma causing sleep disturbance (Aziah, 2014). Improper asthma management may increase the individual's visit to the emergency department and admittance to the hospital and thus, increase their financial burden.

Asthma is a complex disease and its development process involving both central and peripheral airways. There are two major changes that took place in the development of asthma; changes in (1) airway structures and (2) cellular (Saetta and Turato, 2001).

1.3.1 Cellular changes of asthma

Cellular change is a process involves infiltration of inflammatory cells into the airway regions. The pathophysiology of asthma is associated with infiltration of inflammatory cells such as eosinophils, mast cells, lymphocytes, macrophages and neutrophils (Fahy et al., 2000, Saetta and Turato, 2001, Jang et al., 2012, Shifren et al., 2012).

Previous studies agreed that eosinophilic infiltration is considered as the most striking feature for asthma (Fahy et al., 2000). In a review written by Saetta and Turato (2001), the article discussed about the distribution of eosinophilic infiltration in both airways and its physiological implication. In small airways, eosinophils are densely distributed between the smooth muscle and the alveolar attachment of the airway wall which later promotes the airway constriction by decreasing the tethering effects of parenchyma on the airway wall. Meanwhile in the large airways, eosinophils are densely distributed between the smooth muscle and the basement membrane. As a result, smooth muscle shortened and later led to decrease in the airway calibre, thus promoting the airway constriction (Saetta and Turato, 2001).

However, in the case of severe asthma, neutrophils seems to be dominant compared to eosinophils (Monteseirin, 2009). Findings from various study concluded that neutrophilic airway inflammation is more profound in patients of severe asthma compared to patients with slight asthma (Wenzel et al., 1997, Just et al., 2002). Neutrophils as the first cell recruited to the site of allergic reaction may influence the clinical presentation and play a role in development of severe asthma (Monteseirin, 2009). Neutrophilic inflammation is thought to be involved in the progression of persistent airflow limitation in asthma and can lead to sudden death (Shaw et al.,

2007). This is further supported by finding of increased level of neutrophils in the airways and submucous glands from biopsy materials of fatal asthma patients (Sur et al., 1993, Carroll et al., 2002).

1.3.2 Structural changes of asthma

Asthma is usually associated with alteration in the structure of airway, a condition known as airway remodelling, which occurred in conjunction with or because of persistent airway inflammation (Tang et al., 2006, Tagaya and Tamaoki, 2007). Prolonged inflammation leads to alteration in the structure of airway epithelium, mucosal and submucosal regions and contributed to thickening of the airway wall. As illustrated in Figure 1.1 and Figure 1.2, the structural changes observed include epithelial injury including epithelial damage and denudation, goblet cell hyperplasia, mucous gland hypertrophy, increased in the smooth muscle (SM) mass, subepithelial fibrosis, thickening of the airway mucosal, angiogenesis, and enhanced deposition of extracellular matrix (ECM) proteins (Fahy et al., 2000, Mauad et al., 2007, Tagaya and Tamaoki, 2007, Girodet et al., 2011). Airway remodelling involves both small and large airways as well as surrounding of peribronchial areas.

1.3.2 (a) Epithelial cell alteration

Airway epithelium serves as a barrier that protects internal milieu of the lungs from outer environment by isolating the inhaled foreign allergens (Al-Muhsen et al., 2011). In addition, the epithelial also serves as the regulator for both metabolic and immunologic functions in the airways (Tagaya and Tamaoki, 2007). Cells in the epithelium layer are capable to undergo regeneration, allowing normal cell turnover

and restoration of airway and alveolar functions following injury in the lungs (Hermans and Bernard, 1999).

Alteration in the epithelial layer of asthmatic patients including epithelial shedding, loss of ciliated cells, goblet cell hyperplasia, and upregulation of growth factors, cytokines and chemokines (Al-Muhsen et al., 2011, Shifren et al., 2012).

Continuous exposure towards allergens and proinflammatory mediators as in asthmatic cases put the epithelial cells on stress mode. As transforming growth factor-β (TGF-β) gene activated the signalling pathway, the gene induced apoptosis of airway epithelial cells which results in the detachment of the epithelial cells (Halwani et al., 2011).

Histologically, epithelial damage and detachment from the basement membrane has been frequently reported in various asthma models. Increased level of epithelial cells in the bronchoalveolar lavage (BAL) specimens and presence of clustered of sloughed epithelial cells (Creola bodies) in the sputum are correlates with the airway hyperresponsiveness (AHR) (Jeffery, 2001). The degree of AHR is correlates with the degree of epithelial detachment which in turn reflects the severity of the disease (Jeffery, 2001, Aoshiba and Nagai, 2004). However, findings from few studies did not support this correlation. In a review done by Carroll et al. (1993), it was showed the evidence of airways with intact surface epithelium even though in the presence of intense inflammation and other structural changes. This variability is likely due to factors such as inherent fragility of the epithelium in asthmatic, mechanical stresses imposed during bronchoscopy, and the sampling technique (Jeffery, 2001, Shifren et al., 2012).

1.3.2 (b) Mucus secreting cells

Goblet cells and submucosal glands are responsible in secreting mucus into airways which functioning in protecting the epithelial surface from injury. In response to injury, goblet cells become activated and increased in number. Together, a high proportion of goblet cells and submucosal gland enlargement lead to mucus hypersecretion into the airway which is a distinct feature of asthma. Previous studies have consistently shown the correlation of asthma (regardless of disease severity) with goblet cell hyperplasia (Tagaya and Tamaoki, 2007, Shifren et al., 2012). Simultaneously, mucus aggravation also contributed by mucous gland hyperthrophy which are normally found in the subepithelium (Saetta and Turato, 2001, Tagaya and Tamaoki, 2007).

1.3.2 (c) Reticular basement membrane

Asthma is also associated with thickening of subepithelial reticular basement membrane (RBM). Histologically, the epithelial basement membrane consists of two layers namely basal lamina and lamina reticularis. It was reported that thickening of basement membrane in asthma took place in reticularis layer (Tagaya and Tamaoki, 2007). Thickening in this regions occurred due to deposition of collagen type I, III, IV, and ECM proteins. This thickening is also known as subepithelial fibrosis (Jeffery, 2001, Saetta and Turato, 2001, Tagaya and Tamaoki, 2007).

1.3.2 (d) Angiogenesis

Angiogenesis is a process of proliferation of bronchial vessels (BV) which enhance the vascularity of the airways. BV play important role in regulating the airway calibre. Increased in vascularity resulted in vascular congestion and later

caused swollen of the mucosa. As a result, this accounts for narrowing the airway lumen (Fahy et al., 2000, Jeffery, 2001).

1.3.2 (e) Airway smooth muscle

Increased in the smooth muscle mass has been considered as the most prominent feature in development of asthma. In asthmatics, remodelling of SM involved two steps; hyperplasia of myocyte and hypertrophy of myocyte (Jeffery, 2001, Shifren et al., 2012). Hyperplasia of SM was discovered to occur only in the central airways. Whilst, hypertrophy of SM took place in the whole airway tree including bronchioles (Tagaya and Tamaoki, 2007). Previous studies showed that hyperplasia of SM seem to be dominant over hypertrophy with the numbers of myocyte increased two- to three-fold higher than in normal condition (Fahy et al., 2000). Increased in SM resulted in thickening of the airway wall, narrowing of the airway lumen and reduction of the airway calibre in cases of severe asthma (Saetta and Turato, 2001, Aoshiba and Nagai, 2004).

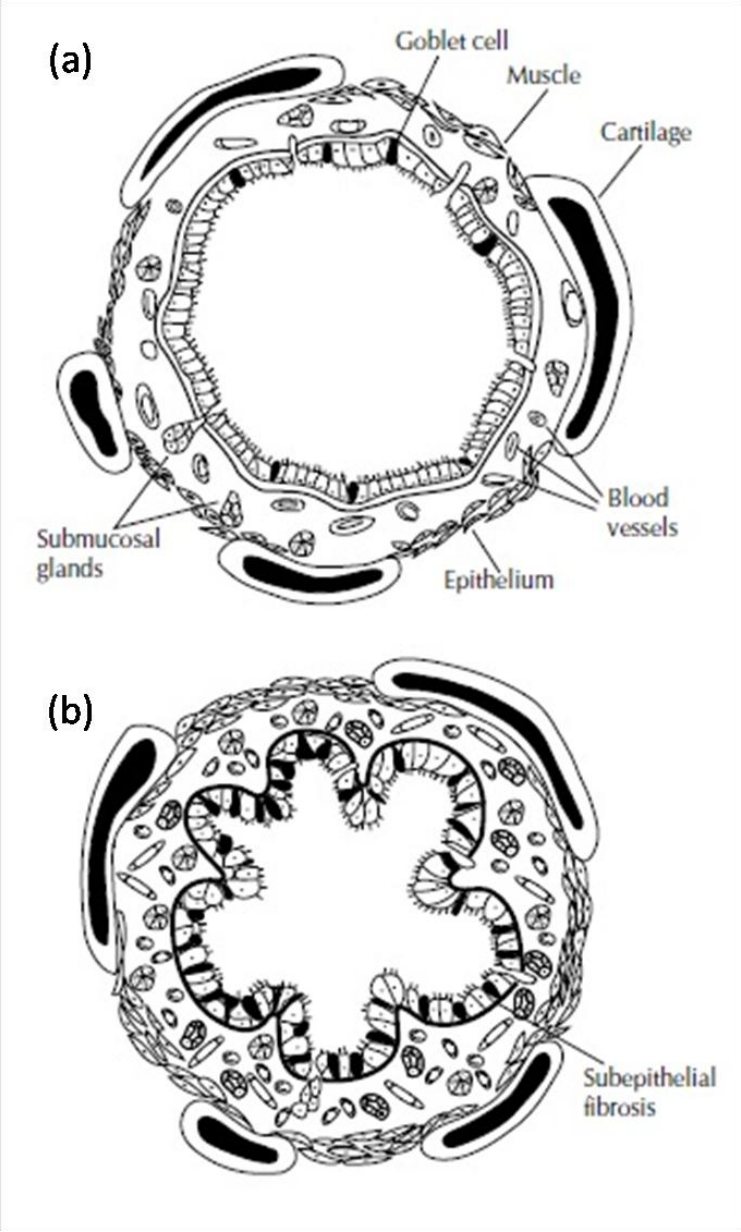

Figure 1.1 Schematic representation of airway remodelling in asthma. (a) the structure of normal and healthy airway while (b) represents the structure of asthmatic airway. Adapted from "Airway Inflammation and Remodeling in Asthma" by Fahy, Corry, and Boushey, 2000, *Current Opinion in Pulmonary Medicine*, 6(1), p. 16.

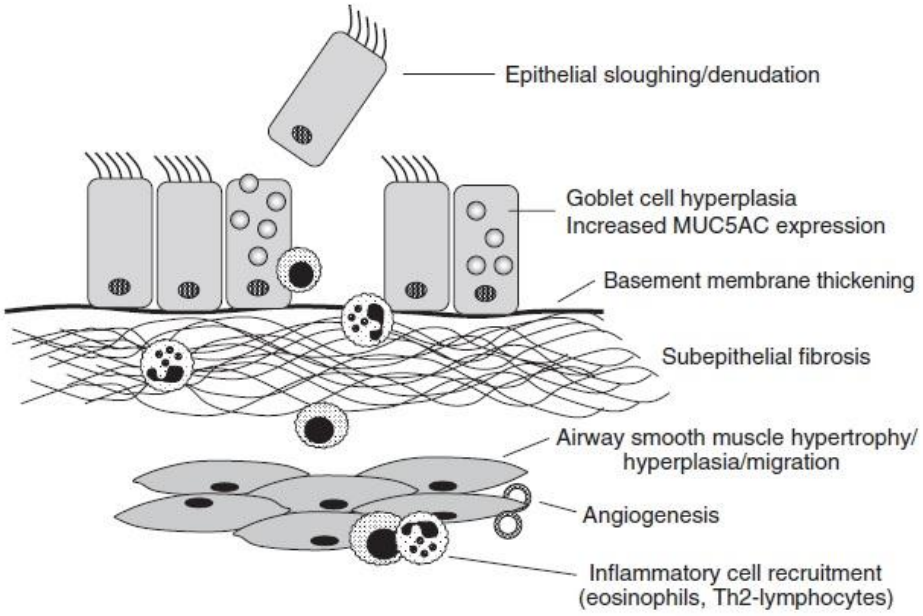

Figure 1.2 An overview of remodelling that took place in airway structure of asthma. Adapted from "Mechanisms of Airway Remodeling in Asthma" by Tagaya and Tamaoki, 2007, *Allergology International*, 56(4), p.332.

1.3.3 Consequences of airway remodelling in asthma

An increased in the SM mass together with angiogenesis and subepithelial fibrosis contributed to the thickening of the airway wall. Thickened airway wall will further narrowing the airway lumen and resulted in significant increase of airway resistance, thus limiting the airflow. Increased in the amount of mucus secretion and the presence of other inflammatory exudates such as plasma protein in the airway lumen resulted in the formation of mucus 'plugs' in the airways. This will further enhance the airflow limitation and hence resulted in airway obstruction (Fahy et al., 2000, Saetta and Turato, 2001, Shifren et al., 2012). All of these consequences are responsible in development of clinical symptoms such as chest tightness, wheezing and breathlessness.

1.4 Treatments in managing lung disease

Lung disease such as asthma and COPD needs immediate treatment to relieve the symptoms once the attack occurred. Currently there are two types of treatment available in managing the disease; conventional drug therapy and alternative therapy.

1.4.1 Conventional drug therapy

Pharmacotherapy remains the most common method in managing lung disease. This therapy has been developed to prevent and control the disease symptoms, reduce the frequency and severity of disease exacerbations and reverse airflow obstructions (Dahl, 2006). By custom, drug therapy was categorized either as bronchodilators (relaxing the airway SM) or as anti-inflammatory drugs (suppressing the airway inflammation) (Fanta, 2009). However, in current practice, these medications are now classified based on their roles in managing the disease – either as quick relieve or long-term control (Fanta, 2009).

Short acting β-agonist (SABA) and short acting muscarinic antagonist (SAMA) are examples of quick relieve medication. Lists of drugs that grouped under SABA type of medications are salbutamol, levalbuterol and meaprotenerol whilst example of SAMA is ipratropium. These drugs are effective in rapid reversal of airflow obstruction and provide prompt relief upon the breathlessness symptoms (Fanta, 2009, Parmar and Raines, 2015).

On the other hand, examples of controller medications used including inhaled corticosteroids (ICS), long acting β-agonist (LABA), long acting muscarinic antagonist (LAMA), leukotriene modulators, and anti immunoglobulin-E (IgE)

therapy (Fanta, 2009, Parmar and Raines, 2015). Of all these therapy, ICS remains as the widely used drug in relieving the asthma attack.

ICS has been considered as the first line therapy in the management of persistent asthma (Dahl, 2006, Patel and Schleimer, 2008). Findings from clinical studies shown that ICS has the ability to significantly reduce the airway inflammation and AHR, preventing the acute exacerbations, improving lung function and decreasing the symptoms severity (Georgitis, 1999, Dahl, 2006). This has been the major factor in the widespread usage of ICS as the frontline in asthma management which also been approved by both national and international guidelines (Dahl, 2006, Patel and Schleimer, 2008).

Despite of positive outcomes of ICS as reported by Dahl (2006) and Georgitis (1999), there were also controversies about the role of corticosteroids in restoring the airway structure. As mentioned earlier, asthma is featured by alteration in the structure of epithelial which includes epithelial damage. Some *in vitro* studies concluded that corticosteroids could increase the apoptosis of epithelial cells which in the case if asthma, this could further contributed to chronic epithelial damage (Dorscheid et al., 2001, Mauad et al., 2007). On contrary, in a retrospective biopsy study, prolong treatment of ICS (10 years) did decreased the inflammation and partially improved the epithelial damage (Lundgren et al., 1988).

It is important to remember that ICS which was developed by chemical constituents also possess side effects to the human beings which are categorized into local side effect and systemic side effect. Examples of local side effects are oropharyngeal candidiasis, dysphonia, reflex cough, bronchospasm and pharyngitis (Dahl, 2006). Local side effects are mostly contributed by deposition of

corticosteroids in the oropharyngeal cavity and usually the effects are dose dependant (Patel and Schleimer, 2008).

Systemic side effects are contributed by absorption of corticosteroids through lungs and gastrointestinal tract (GIT). Most of the currently available ICS have been designed to undergo first-pass metabolic inactivation in the liver following absorption from the GIT before reaching the systemic circulation (Wood and Barnes, 1995, Fanta, 2009). Nevertheless, prolong administration of ICS and high dose therapy can cause adverse side effects such as skin thinning and skin bruising, cataracts, elevated intraocular pressure, accelerated loss of bone mass, bone fractures and osteoporosis (Dahl, 2006, Fanta, 2009).

Due to adverse side effects possess by conventional drug therapy especially ICS, this highlights the need to find alternative therapy with less side effects preferably nature-based therapy.

1.4.2 Alternative treatment

Alternative treatments from natural resources could provide promising results in finding solutions to replace conventional therapy and thus minimizing the risk of gaining unwanted secondary effects from the treatment itself. For this purpose, various studies have been carried out in examining the potential ability of various natural herbs in reducing and managing the disease symptoms. Several studies concludes that extracts from *Nigella sativa* oil (El Gazzar et al., 2006, Balaha et al., 2012), *Mimosa pudica* plant (Yang et al., 2011b), *Lagerstroemia indica* plant (Yang et al., 2011a), *Duchesnea chrysantha* herb plant (Yang et al., 2008), *Bambusae caulis* in Taeniam (family of bamboo) (Ra et al., 2010) and skullcapflavone II, a flavanoid

isolated from *Scutellabaria baicalensis* herb plant (Jang et al., 2012) have the ability to ameliorate ovalbumin (OVA)-induced airway inflammation in animal model of asthma. This include reducing the eosinophils infiltration, inhibiting the goblet cell hyperplasia, improving the lung functions and suppressing the level of T-helper type (Th2) cytokines and thus restoring the balance of Th1/Th2 cytokines.

1.5 Honey – the hidden forest gold

Honey is a natural product produced by honeybees from nectars. By nature, honey is a viscous substance with pleasant taste as it is composed by a complex mixture of sugars. The composition and physicochemical properties of honey are variable according to its geographical origin, floral source, environmental and seasonal conditions processing techniques (Erejuwa et al., 2012a). Honey can be grouped into monofloral or multifloral depending on its botanical nectar source. Monofloral honey is produced by nectars collected from one flower species whilst multifloral honey is produced by nectars collected from multiple flower species (Erejuwa et al., 2012a).

Honey has been scientifically reported to have both medicinal and nutritional values based on its various biological properties including as anti-inflammatory, antioxidant, anti-bacterial, anti-diabetic, anti-microbial and wound healing (Ahmed and Othman, 2013). Manuka honey, a monofloral honey originated from New Zealand, has been extensively studied and is well known for various medicinal properties and its antioxidants, anti-bacterial, and anti-fungal properties has been well published. In fact, Manuka honey has been setup as the ‘gold standard’ for comparison studies with other types of honey (Ahmed and Othman, 2013).

Malaysia is well known for its varieties of honey such as Tualang honey, Gelam honey, Kelulut honey, and Acacia honey. Among all types of Malaysian honey, Tualang (*Koompassia Excelsa*) honey (TH) recently gained attention from the researchers to further explore its potential benefits.

TH is a multifloral honey produced by the rock bee (*Apis dorsata*). TH gained its name as the honey is collected from hives build up in the branches of *Koompassia excels*a plant or locally known as Tualang tree (Ahmed and Othman, 2013). On the other hand, Gelam honey is also produced by *Apis dorsata* bees in which nectar and pollens are collected from the plant *Melaleuca cajuputi Powell* or locally known as Gelam tree (Khalil et al., 2011). Acacia honey gained its name as the nectar is collected by *Apis mellifera* bees from the *Acacia mangium* trees (Moniruzzaman et al., 2013).

1.5.1 Composition and physicochemical properties of Tualang honey

In general, honey is composed of mixture of sugars mainly fructose (38%) and glucose (31%), and contains more than 180 components phenolic acids and flavonoids, enzymes, vitamins, minerals and proteins (Viuda-Martos et al., 2008, Ahmed and Othman, 2013). TH has dark brown appearance with pH ranging from 3.55 to 4.00 and a specific gravity of 1.335 (Ghazali, 2009). The low pH of TH is similar with Manuka honey but more acidic compared to both Kelulut and Gelam honey, thus make it effective as antibacterial and antimicrobial against several pathogenic microorganisms (Tan et al., 2009a, Ahmed and Othman, 2013).

Analysis of volatile compounds in TH using gas chromatography mass spectrometry revealed that TH is primarily composed by 58.54% of hydrocarbons,

11.26% of ketones, aldehydes (10.26%), acids and alcohol (6.76%), and organic acids (1.44%). Other constituents including terpenes and furans (Nurul et al., 2013).

A total of six phenolic acids have been discovered in TH which are gallic acid, syringic acid, benzoic acid, transcinnamic acid, p-coumaric acid and caffeic acid. TH also consists of five flavonoids including catechin, kaempferol, naringenin, luteolin and apigenin (Khalil et al., 2010, Khalil et al., 2011, Ahmed and Othman, 2013). Phenolic compounds has been reported to exhibit properties such as antioxidants, anti-inflammatory, anti-carcinogenic and others (Kassim et al., 2010).

1.5.2 Medicinal benefits of Tualang honey

Honey has been used to cure various kinds of ailments in both traditional and modern medicine. To date, various studies have been conducted in both *in vivo* and *in vitro* to investigate the biological properties of honey especially TH. TH is well known to possess properties such as anti-inflammatory (Bashkaran et al., 2011), anti-bacterial (Tan et al., 2009a, Tan et al., 2009b), anti-diabetic (Erejuwa et al., 2012a), anti-cancer (Ghashm et al., 2010), anti-hypertensive (Erejuwa et al., 2012b) and antioxidant (Ahmed and Othman, 2013).

It has been well documented that TH can serve as alternative treatments in the wound healing. Both clinical and pre-clinical studies show the promising results of application of TH on wound healing. In a clinical study done by Imran and her friends, application of TH-based hydrogel improved the wound healing in the patients of split-skin graft (Imran et al., 2011). This is further supported by findings from other clinical study which reported the ability of TH dressing in healing the diabetic foot wounds (Nawfar et al., 2011), incision wound (Zaharil et al., 2011), and

burn wounds (Khoo et al., 2010, Sukur et al., 2011). In general, honey improves wound healing by abating oedema, inflammation, exudation and promoting the growth of epithelial cells and fibroblast (Ahmed and Othman, 2013). The ability of TH in wound healing is attributed by its antibacterial and antioxidant properties (Tan et al., 2009a, Nasir et al., 2010). High content of phenolics and flavonoids in TH attributed to its antioxidant activities which is higher compared to other local and commercially available honey (Khalil et al., 2011).

Although honey has high level of fructose and glucose, however, it can be used in managing the diabetes mellitus (DM). In an *in vivo* study, combination of TH with metformin or glibenclamide (common drug to treat DM) significantly lowered the blood glucose level compared to the metformin or glibenclamide alone. Hence, this proves the ability of TH in combination with hypoglycaemic agents to improve the glycaemic control in streptozotocin-induced diabetic rats (Erejuwa et al., 2011).

1.5.3 Honey as treatment for respiratory diseases

The scientific findings that reported the potential use of honey in treating respiratory-related diseases are very limited. So far, there are three published works that studied the effect of honey in treating cough and upper acute asthma.

Cough is usually associated with upper respiratory infections. Honey supplementation has been widely used to treat cough since ancient times. Previous study supports the goodness of buckwheat honey in comparison to dextromethorphan as treatment in children with nocturnal cough and sleep difficulty due to upper respiratory tract infection (Paul et al., 2007). A preliminary study to assess the benefit of TH in reducing acute respiratory symptoms such as cough, rhinitis and

sore throats was conducted in 2007 (Sulaiman et al., 2011). Studied population was Malaysian hajj pilgrims who were commonly associated with these symptoms while performing hajj. Oral consumption of honey twice daily did aid in alleviating the respiratory symptoms among pilgrims. Due to a small sample size (n=56) and a short study timeline (42 days), thus, the significant effect of TH in reducing respiratory symptoms could not be concluded (Sulaiman et al., 2011).

Other study done by Maksoud and Rahman (2007), reported honey inhalation was effective in treating acute bronchial asthma in paediatric patients. This is the only study that focused on the effect of honey in treating lung injury. However, the mechanism of action of honey as lung injury treatment remains unclear. The authors did not mention the effect of honey on pathogenesis of lung injury at both cellular and molecular level. In fact, none of the documentation did report the potential use of TH in lung injury management so far. This later became the subject of interest in this current study.

1.6 Rabbits as animal model of chronic lung injury

As mentioned earlier, chronic lung injury contributed to thousands of death annually worldwide. Thus, development of prevention strategies and new treatment methods for the disease is a must. Therefore, a biological model is needed in order to understand the mechanism and development of a disease at both cellular and molecular level. Animal model seem to be a perfect experimental model as it provides an experimental setting that allow the researchers to study the interaction between immune system and the functioning respiratory system (Kamaruzaman et al., 2013).

An ideal animal model of chronic lung injury must have the ability to reproduce both the anatomical and physiology features resembling to the human chronic lung disease. This includes IgE-mediated sensitivity to antigens, increase airway resistance, acute bronchoconstriction, chronic airway inflammation, eosinophilic infiltration, mucus hypersecretion, Th2 cytokines production, airway wall remodelling and smooth muscle hyperplasia (Keir and Page, 2008). Choosing an ideal animal model for chronic lung disease is very decisive and need critical judgement. It is important to choose animals that have similarity with the physiology and organ size of human lungs which can only be offered by large-sized animals (Zosky and Sly, 2007). Measurement of lung functions in small-sized animals would seem to be technically challenging. Besides that, the anatomical structure of the lungs also an important criterion to be considered in choosing the right animal model.

Rabbit shared similarities with human airways and is phylogenetically closer to human than any other rodents. Both rabbit and human have abundant submucosal gland and mucous secreting goblet cells in the upper airways in contrast to mice (Koehler et al., 2005). This provides an advantage in the disease development and research as asthma is also associated with goblet cell hyperplasia. Moreover, the model itself possess lungs that are large enough to study lung mechanics (Kamaruzaman et al., 2013). Thus, this provides rabbit as a useful species to study lung biology in order to understand the human lungs conditions. This is supported by earlier work in rabbits as model in investigating the effectiveness of lung reduction surgery in treatment of emphysema and COPD (Keir and Page, 2008). The treatment is a success and currently being used at the clinical setting. It similarities with human lungs provides rabbits as useful tool as models for studying asthma.

1.7 Allergen used in inducing chronic lung injury

In any animal model of allergic disease, it is important to develop the disease which having similar immunological and pathophysiological characteristic as in human. This can be achieved by the use of allergen such as ovalbumin (OVA), house dust mite (HDM) and *Aspergillus fumigatus*.

1.7.1 Ovalbumin

OVA is the most commonly allergen used in inducing allergic disease in animal model. OVA is usually being introduced into the model system by two steps; sensitization and repeated exposure by means of inhalation. Sensitization of OVA is usually coupled with Th2-skewing adjuvant such as aluminum hydroxide (alum) with the aim to prime the allergic airway inflammation (Conrad et al., 2009). Both OVA and alum are introduced into the animal model by injection into the peritoneal cavity. Repeated injections of OVA together with alum is reported to induce strong, sustained sensitization with a preferential Th2-type immune response and production of allergen-specific IgE and immunoglobulin G1 (IgG1) which resembling the allergic sensitization in atopic individuals (Hamelmann and Gelfand, 1999). Following sensitization, animals models are further exposed to repeated inhalation of OVA which later develop AHR and recruitment of inflammatory cells such as eosinophils, T-cells and neutrophils into airway tissue (Piavaux et al., 2007).

1.7.2 House dust mite

HDM is an aeroallergen and one of the examples is *Dermatophagoides pteronyssinus*. HDM is quite dangerous compared to OVA as exposure to HDM by any genetic susceptible individuals can cause symptoms from as mild as atopic

dermatitis to bronchial asthma (Piavaux et al., 2007). Unlike OVA which require the presence of adjuvant, intranasal exposure towards HDM alone can generate both acute and chronic airway inflammation, induce the development of AHR and increase the Th2-type inflammatory cell response (Cates et al., 2004, Johnson et al., 2004).

1.7.3 *Aspergillus fumigatus*

A.fumigatus is an allergic fungi which are widely distributed in the environment. Sensitization by *A.fumigatus* causing elevated levels of IgE and eosinophils, enhances the Th2 cytokines, development of AHR and profound airway remodelling (Piavaux et al., 2007). The crude extract of *A.fumigatus* is highly antigenic and it contains active substances such as ribotoxins and protease. Sensitization with *A.fumigatus* do not required adjuvant as protease itself serves as adjuvant by inducing epithelial damage in the airways and allowing normally excluded antigens to bypass the mucosal barrier (Tomee et al., 1997).

1.8 Rational of the study

The establishment of aerosol delivery of honey using nebulizer was aimed to provide an alternative option for treatment of chronic lung injury by using nature-based product. With the increase in the awareness of side effects offered by the conventional drug therapy, it is hoped that honey-based medication could be a potential as a complementary and alternative medicine for the management of chronic lung injury patients.

1.9 Objectives of the study

1.9.1 General objective

The general objective of this study was to study the effect of aerosolised-honey on airway epithelium repair and regeneration following ovalbumin-induced chronic lung injury in rabbit model.

1.9.2 Specific objectives

This study was aimed to address the following objectives:

1. To develop rabbit as an *in vivo* model of chronic lung injury (i.e. that mimic human condition of asthma) by repetitive exposure to OVA.
2. To determine the effective dose of honey administration to the chronic airway using aerosol-based inhalation technique.
3. To study the effect of aerosolised-honey on pathophysiological changes of chronic-induced lung airway tissue (i.e. airway epithelium, mucosal region, submucosal region, goblet cells) using established histological staining techniques.

1.10 Study hypothesis

1. Inhalation of honey can reduce the inflammatory responses of the airway.
2. Aerosolisation is feasible and effective route of delivery of honey for treatment of chronic lung diseases.