# THE EFFECT OF VIRGIN COCONUT OIL (VCO) ON AIRWAY INFLAMMATION AND MUCUS SECRETING CELLS IN AN ANIMAL MODEL OF CHRONIC LUNG INJURY

**by**

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**Thesis submitted in fulfilment of the requirement for the Degree of**

**Master of Science**

**JANUARY 2015**

## DEDICATIONS

This thesis is dedicated to my father, Kamalaldin Abd. Latif, my mother, Rohani Zakaria and my lovely sisters and brothers who have been a great source of my inspiration and motivation.

## ACKNOWLEDGEMENT

All works done in this thesis will be impossible to be completed without chances given by Allah SWT, by giving me strength, patience, knowledge in completing this battle to the end. I thank HIM for the most beautiful blessing throughout this journey.

My expression of grateful was to my supervisor, Dr. Badrul Hisham Yahaya, who has guided me along this thesis progression until I able to complete in time. My appreciation towards his knowledge, patience, guidance, endless encouragement in encouraging me in performing the research and writing this thesis. Special thanks to Dr.Gurjeet for her kindness for helping me with the consultation regarding the histological slides and Prof. Dr. Siti Amrah binti Sulaiman for being my co-supervisor. Warmest appreciation to my fellow labmates, Egi Kardia, Nurfatin Asyikhin Kamaruzaman, Ahmad Zaeri Latahir, Nurshuhaidatul Sarmiza, Nazilah Abd Satar, and Norashikin Zakaria and all Animal Research Centre staff for being helpful and guiding me during the period of my study.

Heartfelt thanks for my parents and siblings, in their support and courage for almost two years of my study. This meaningful appreciation also goes to my dearest friend I made in my life, Ahmad Thaifur Khaizul, Siti Hasnidar Harun, Mohd Syarifuddin Mat Zali, and Abdul Rahman Zainal who has always being right beside me through up and down as a student and giving me moral support. Thank you for always believing in me and encouragement shown with lots of love and support.

Last but not least, to the MOSTI who providing a research grant; ScienceFund grant (305/CIPPT/613224) in supporting the funding of my research work.

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| | |
|---|---|
| ATI | Alveolar type I |
| ATII | Alveolar type II |
| ARDS | Acute respiratory distress syndrome |
| ALI | Acue lung injury |
| APCC | Asian and Pacific Coconut Community |
| AB-PAS | Alcian Blue-Periodic Acid Schiff |
| AHR | Airway hyperresponsiveness |
| BAL | Bronchioalveolar lavage |
| BSA | Bovine serum albumin |
| COPD | Chronic Obstructive Pulmonary Disease |
| CDK2 | Cyclin dependent kinase 2 |
| EI | Electron impact |
| ELISA | Enzyme-linked immune-sorbent assay |
| ECM | Extracellular matrix |
| FOXA2 | Forxhead box A2 |
| GCH | Goblet cell hyperplasia |
| GC-MS | Gas chromatography mass spectrometry |
| He | Helium |
| H&E | Haematoxylin & Eosin |
| HCl | Hydrocholric acid |
| HRP | Horse-raddish peroxidase |
| IgE | Immunoglobulin E |
| IL1-18 | Interleukin 1-18 |
| IL4 | Interleukin 4 |
| IL5 | Interleukin 5 |

| | |
|---|---|
| IL9 | Interleukin 9 |
| IL10 | Interleukin 10 |
| IL13 | Interleukin 13 |
| i.p. | Intraperitoneal |
| i.m. | Intramuscular |
| IHC | Immunohistochemistry |
| LABA | Long-acting beta agonist |
| MUC | Mucin |
| MUC5AC | Mucin5AB |
| MUC5B | Mucin5B |
| MUC1 | Mucin1 |
| MUC2 | Mucin2 |
| MUC4 | Mucin4 |
| MUC7 | Mucin7 |
| MUC8 | Mucin8 |
| MUC13 | Mucin13 |
| MUC15 | Mucin15 |
| MUC19 | Mucin19 |
| MUC20 | Mucin20 |
| MARCKS | Myristoylated alanine-rich C protein kinase substrate |
| MMAD | Mass median aerodynamic diameter |
| MMP9 | Matrix metalloproteinase 9 |
| NMUS | National Medicine Use Survey |
| NHMS III | Third National Health and Morbidity Survey |
| OVA | Ovalbumin |
| PCNA | Proliferating cell nuclear antigen |
| PBS | Phosphate buffer saline |

| | |
|---|---|
| RBD | Refined, Bleached and Deodorized |
| STAT6 | Signal Transducer and Activator of Transcription 6 |
| SPDEF | SAM pointed domain ETS factor |
| SABA | Short-acting beta agonist |
| TGF-α | Transforming growth factor- α |
| TGF- β | Transforming growth factor-β |
| $Th_1$ | T helper type 1 |
| $Th_2$ | T helper type 2 |
| UK | United Kingdom |
| VCO | Virgin Coconut Oil |

## LIST OF SYMBOL

| | |
|---|---|
| % | percent |
| µL | microliter |
| µm | micrometre |
| °C | degree of celcius |
| cm | centimetre |
| mm | micrometre |
| kg | kilogram |
| µg | microgram |
| mg | miligram |
| $log_{10}$ | logarithm 10 |
| ® | registred |
| ™ | trademark |
| mL | millilitre |
| eV | electron voltan |
| m/s | millimetre per second |
| mM | millimolar |
| pg | picogram |

## LIST OF PUBLICATIONS AND PRESENTATIONS

### Presentations

Kardia, E., **Kamalaldin, NA**., Yusoff, NM., Z, Zakaria., Yahaya, B. Vacuolation as an immediate cellular response towards adaptation of skin- and bone marrow-derived fibroblast-like cells in airway culture media. *International Society for Cell and Gene Therapy of Cancer*, Singapore, 4-6th of October 2012.

**Kamalaldin NA**, Sulaiman SA, Seeni A, and Yahaya BH. Virgin coconut oil (VCO) inhibits cell growth via apoptosis on lung cancer cell lines. *Natural Product Conference, Shah Alam,* 13-15th of March 2013.

### Publications

Kamaruzaman NA, Kardia E, **Kamalaldin NA**, Latahir AZ, and Yahaya BH. The rabbit as a model for studying lung disease and stem cell therapy. *Biomed Reseacrh International.* Vol. 2013, Article ID 691830, 12 pages, 2013. doi:10.1155/2013/691830. (IF: 2.880).

**Kamalaldin NA**, Sulaiman SA, Seeni A, and Yahaya BH. Virgin coconut oil (VCO) inhibits cell growth via apoptosis on lung cancer cell lines. *The Open Conference Proceedings Journal.* doi: 10.2174/2210289201304010289.

Dewan Kosmik. Julai 2013. **Nurulain 'Atikah Kamalaldin**. Teknologi: Minyak Kelapa Dara Rencat Sel Kanser.

**Kamalaldin NA**., Sulaiman SA, Yahaya BH. Apoptosis and morphological changes in lung cancer cells induced by exposure to virgin coconut oil. *Submitted for publication.*

**KESAN MINYAK KELAPA DARA KE ATAS INFLAMASI SALUR PERNAFASAN DAN SEL REMBESAN MUKUS DI DALAM MODEL HAIWAN KECEDERAAN PARU-PARU KRONIK**

# ABSTRAK

Peningkatan kadar kekerapan penyakit asma telah menjadikan penyakit ini tersenarai sebagai nombor tiga di antara penyakit berisiko tinggi di seluruh dunia. Sedutan terapi dadah yang sering di beri pada masa kini bagaimanapun telah dilaporkan dengan kesan sampingan yang teruk, terutama pada fungsi paru-paru dengan penggunaan jangka masa panjang. Dalam kajian ini, minyak kelapa dara telah diberikan kepada model haiwan asma untuk membuat pemerhation terhadap kesan sedutan minyak kelapa dara pada penyusupan sel inflamatori salur pernafasan, pembentukan semula struktur saluran udara (ketebalan epitelium, mukosa dan submucosa), hiperplasia sel goblet dan aktiviti proliferasi sel epitelium setelah didedahkan kepada alergen. Arnab telah disensitifkan dengan suntikan gabungan ovalbumin dan alum dan diikuti dengan sedutan ovalbumin. Ini bertujuan untuk mencetuskan serangan asma. Model asma kemudiannya diberikan rawatan dengan minyak kelapa dara secara sedutan melalui laluan menyelamat (minyak kelapa dara sebagai kumpulan ejen penyelamat) dan laluan pencegahan (VCO sebagai kumpulan ejen pencegahan). Analisis GC -MS untuk mengenalpasti sebatian yang mempunyai kebolehan sebagai anti-radang dan anti-asma juga telah dijalankan. Keputusan menunjukkan pengurangan ketara dalam kumpulan menyelamat dari segi infiltrasi sel radang, ketebalan struktur saluran udara (epitelium dan mukosa) dan bilangan sel goblet. Pengurangan juga diperhatikan pada aktiviti proliferasi sel epitelium saluran udara. Dalam kajian ini, analisis GC-MS telah menunjukkan bahawa terdapat dua

komponen yang mungkin menyumbang kepada keberkesanan rawatan minyak kelapa dara secara sedutan, iaitu asid laurik (40.86 %) dan asid *capric* (5.17 %). Secara kesimpulan, kajian ini menunjukkan bahawa kedua-dua kumpulan rawatan minyak kelapa dara mempunyai potensi sebagai ubat anti-asma. Walau bagaimanapun, pemerhatian yang lebih baik dapat ditunjukkan dalam kumpulan penyelamat, dan ini dapat disimpulkan bahawa minyak kelapa dara adalah lebih sesuai untuk bertindak sebagai ubat bagi melegakan atau mengurangkan serangan asma, tetapi tidak boleh mencegah tercetusnya asma semasa pemekaan alergen pada saluran udara. Walau bagaimanapun, kajian mendalam pada masa hadapan perlu dilakukan untuk menentukan laluan di mana minyak kelapa dara mampu mengurangkan tindak balas sel dalam alahan asma,

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## ABSTRACT

A high prevalence of asthma over the years make the disease become a number three among list of live-risked disease worldwide. Inhalation of current drug therapies however has been recorded with severe side effects on the lung function, due to long term usage. In this study, virgin coconut oil (VCO) was given to animal model of asthma as to observe the effect of the inhalation of VCO on the infiltration of airway inflammatory cell, remodelling of airway structures (epithelium, mucosa and submucosa thickness), goblet cell hyperplasia and the proliferation activity of airway epithelial cells following the allergen exposure. The rabbits were elicited with combination of ovalbumin and alum injection and followed with ovalbumin inhalation, in order to induce the onset of asthma. The asthma model was then given the treatment with VCO-inhalation via rescue route (VCO as rescue agent group) and preventive route (VCO as preventive agent group). The GC-MS analyses for possible anti-inflammatory and anti-asthmatic compounds were also carried out. Result showed a significant reduction in the rescue group in terms of infiltration of inflammatory cell, thickness of airway structure (epithelium and mucosa) and goblet cell numbers. Reduction was also observed on the proliferation activity of the airway epithelial cell. In this study, the GC-MS analyses showed there were two possible components that might contribute to the effectiveness of VCO-inhalation, which was lauric acid (40.86%) and capric acid (5.17%). As a conclusion, the study showed that both treatment groups exhibited promising result for the VCO as anti-asthmatic

remedies. However, better outcome was observed in rescue group, which can be concluded that the VCO was more suitable to act as medication in relieving or reducing the asthma attacks, but not to prevent the asthma onset during allergen sensitization in the airway. However, extensive future study needs to be carried out in order to determine the pathway in which the VCO was able to reduce the cellular response of allergic asthma.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Overview

Asthma is an airway disorder that is characterised by a specific pattern of inflammation that is largely driven via immunoglobulin (Ig) E-dependent mechanisms in the airway (Barnes, 2008). The pathophysiology of asthma is complex and involves airway inflammation, intermittent airflow obstruction and bronchial hyperresponsiveness. During the attacks, swelling of the airway smooth muscle or smooth muscle hypertrophy limits the airflow, thus reduces the amount of air that can pass by (Barnes, 2008, Bentley and Hershenson, 2008). In asthma patients, common signs and symptoms develop during asthma attack are chest tightness, coughing, wheezing and difficulty in breathing. Asthma onset can be triggered by internal and external factors, which are environmental allergens, animal hair, dust, viral respiratory tract infections, exercise, irritants, emotional factors or stress, cigarette smoke and changes in weather (Keir and Page, 2008, Hernandez et al., 2012).

According to asthma association in United Kingdom (UK), asthma is extremely common and affects people regardless of ages and social background. In the UK, one in every five (1/5) household is exposed to asthma onset in their lifetime. It is estimated that 5.4 million of UK population are currently treated for asthma, which 4.3 million is adults and the remaining is children with known diagnosis of asthma (Asthma Association of UK). Three hundred million people worldwide also have

been estimated to have asthma with one for every 250 death. According to National Medicines Use Survey (NMUS) of Malaysia in 2006, asthma was ranked the 3rd highest amongst chronic disease as reported in the Third National Health and Morbidity Survey (NHMS III). In clinical treatment for asthma, glucocorticoid is the most used drug for relieving asthma symptoms in patients. However, according to Weiner et al. (1992), long term treatment with glucocorticoids in higher doses may cause inspiratory muscle weakness (Weiner et al., 1992).

Recently, the trends of using natural-based products as an alternative and complementary medicine for treatment of critical diseases like cancer are growing over time. There are many researches that using plant-based materials to block the possible route to disease onset (Popova et al., 2005, Gao et al., 2011, Althunibat et al., 2013). Anti-asthmatic and anti-inflammatory effect of several plant-based therapies has been done by several researchers, where they look into the effect of thymoquinone (TQ) from *Nigella sativa'*s seed, curcumin from *Curcuma longa* L, marine red alga (*Laurencia undulate*), Bambusae Caulis in Taenim extract and *Lagerstroemia indica* on animal model of chronic and acute airway inflammation (El Gazzar et al., 2006, Jung et al., 2009, Ra et al., 2010, Yang et al., 2011, Karaman et al., 2012). A study by Yang *et.al* (2008) which used *Duchesnea chrysantha* (Dc) to examine the effect of extract on leukocyte infiltration and mucus secretion reported that the Dc extract reduced the inflammation due to leukocyte infiltration and mucus hyperproduction by goblet cells (Yang et al., 2008). Other than that, anti-inflammatory activities of *Cinnamomum cassia* (Cc) in LPS-model of mouse has been conducted by a team from Taiwan (Liao et al., 2012), where they found out that cinnamic aldehyde (Cs constituent) was able to increase the activities of antioxidant enzymes.

## 1.2 Lung Disease

Lung disease or also known as respiratory disease is the commonest medical condition worldwide, as reported in United States, Canada and also Malaysia. The contribution to the high medical record, statistics can be due to the smoking habits, infections as well as the genetics factors (Siddiqui and Brightling, 2007, Vercelli, 2008, Weiss et al., 2011, Lau et al., 2012). Normal and healthy lung conditions provides sufficient forces in inhaling oxygen and expelling carbon dioxide into and out from the lung. However, abnormalities in lung function which involve changes on the normal structures of the airway tree resulting in difficulties in breathing and other life-threatening cases such as asthma, chronic obstructive pulmonary disease (COPD), infectious lung diseases, lung cancer, tuberculosis and cystic fibrosis.

## 1.3 Chronic Lung Disease

Chronic lung disease or chronic respiratory diseases are commonly used terms to refer to chronic lung diseases such as COPD, asthma, chronic bronchitis and emphysema (Jeffery, 2001, Goldklang et al., 2013). These diseases are worsening over time and cause difficulties in patients living with the circumstances. Under such conditions, chronic lung disease can cause narrowing of the airways lumens (Figure 1.1) which in turn limiting the volume of air in and out from lungs and causes shortness in breathing. Patients living with chronic lung disease often coughing, produce more mucus and having shortness in breath. These symptoms will develop after several years living with unhealthy environment and lifestyle, such as smoking and air pollution, especially in industrial areas (Van Lieshout and MacQueen, 2008).

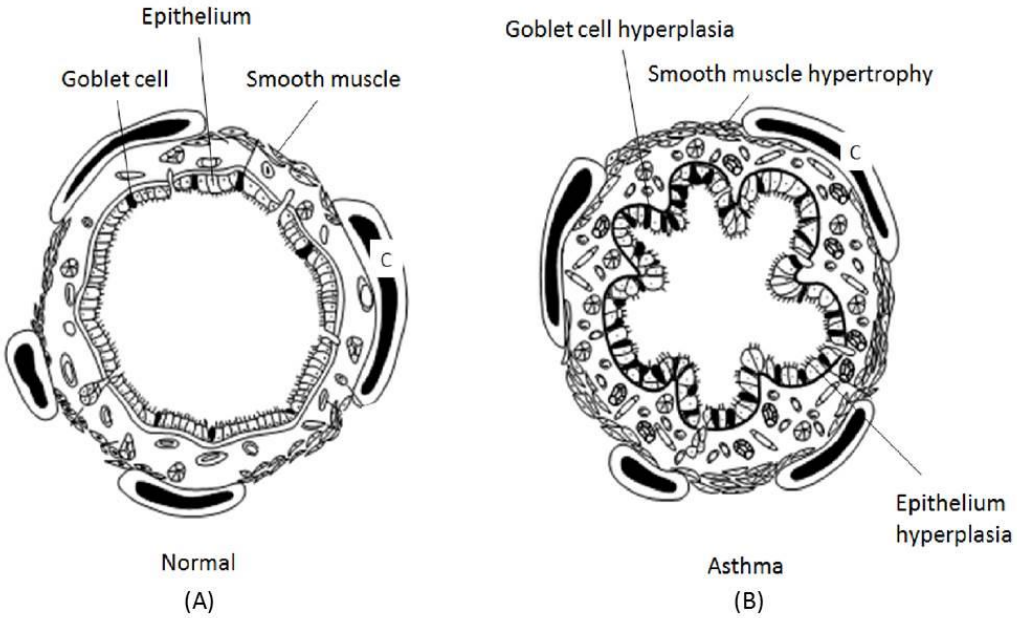


**Figure 1.1** Normal airway (A) vs asthmatic airway (B). Airway in normal condition permits sufficient exchange of air during inhalation and exhalation proses (A). The airways of chronic lung disease (asthmatic airway) (B) are inflamed due to the epithelium hyperplasia, goblet cell hyperplasia, smooth muscle hypertrophy which in turn blocks the airway passage, causing difficulties and shortness in breathing (Adapted and modified from Fahy, Corry, & Boushey, 2000). *Keyword: C-cartilage.*

### 1.3.1 Asthma

Asthma is a chronic inflammatory disorder of the airways which involves numbers of inflammatory cells such as mast cells, eosinophil and T lymphocytes. From a pathologic standpoint, asthma is characterized by a combination of hyperplasia and hypertrophy of the smooth muscle bundle in both small and large airways (Woodruff et al., 2004, Zhang et al., 2004). In asthma patients, the inflammation action of the cells may cause repeated wheezing, breathless, chest tightness and cough. The symptoms particularly take place at night and/or in the early morning. The changes in temperature of the air serve as external stimulus in asthma. Smoking, one of the common lifestyle nowadays, also can contribute to asthma onset as being reported in a research by Goldklang et.al (2013).

Cigarette smoke, home mite and dust is the commonly external stimuli that can activate series of action of gene expression that later induces asthma onset (Townley and Horiba, 2003, Schraufnagel et al., 2010). The stimuli detected by receptor on cells will activate the action of $Th_2$ lymphocytes which will produce inflammatory cytokines. Inflammatory cytokines involve in asthma is interleukin (IL) 4, 5, 9 and 13. The actions of these cytokines will inflame the airway muscle thus remodel the airway structure and cause blockage of respiratory process (Zhen et al., 2007, Krane et al., 2009). The airway lumen is blocked by mucus overproduction by goblet cells that consist of plasma protein from airway vessels and mucus glycoprotein by goblet cells hyperplasia (Hallstrand et al., 2012).

In airway remodelling, there are several changes occurs during the process. The changes involved epithelial goblet cell hyperplasia and metaplasia, collagen deposition and thickening of the lamina reticularis, smooth muscle hyperplasia, and proliferation of airway blood vessels (Davies et al., 2003). Hyperplasia is the process of increasing the goblet cells numbers while metaplasia is the ability of Clara cells and nonciliated cells to transform into goblet cells after sensitization occur (Curran and Cohn, 2010).

## 1.4 Cellular Arrangement of the Lungs

Lung consists of several cell types that line up the inner layer of the airway cavity. During the process of lung morphogenesis and after acute and chronic lung injury, the cells that build up the lungs differs due to the numbers, localization, and function of each cell niches vary dynamically (Cardoso and Whitsett, 2008) Airway epithelial cells of the lungs, such as alveolar type I (AT I), alveolar type II (AT II), nonciliated cell (clara cell), basal cell, goblet cells and also ciliated cells were differentiate and

interact with each other in maintaining the cellular composition of the respiratory system (Park et al., 2006, Cardoso and Whitsett, 2008, Franks et al., 2008). In mature lung, the lung surface is equipped with the nonproliferative, slow rates of mitosis, and prolonged survival of the resident cells (Cardoso and Whitsett, 2008). Most of the cells that can be found in normal lung are alveolar type I, alveolar type II and also clara cell, and these cells serve as the progenitor cells of the airway epithelial and actively involved in repair process during the lung injury (Kotton and Fine, 2008, Liu and Engelhardt, 2008, Rawlins, 2008). After infection, inflammation or exposure to toxic substances, the proliferation and differentiation capability of the progenitor cell will be activated. Alveolar type I which cover about 95 percent of the alveolar surface area is the squamous epithelial cells which responsible in forming the alveolar wall and will be degenerated during injury (Dobbs et al., 1998, McElroy and Kasper, 2004, Wong and Johnson, 2013). Unfavourable condition to the airway exchange process will turn on the repair pathway in alveolar type II. Alveolar type II, which is the progenitor cell to alveolar type I will be proliferated and differentiate rapidly into alveolar type I cells, thus replacing the injured cells (Figure 1.2). Alveolar type II (ATII) cells are cuboidal cells that made up the remaining 5 percent of the alveolar wall (Wu and Wei, 2004, Akram et al., 2013). Alveolar type II cells function in secreting the surface surfactant, control fluid levels and replace injured cells during exposure to the toxicant substances (Zheng et al., 2013). Other than alveolar type II, nonciliated columnar and basal cells can proliferate into other respiratory cells, such as Clara cells, goblet cells and also ciliated cells. Goblet cells are mainly involved in the mucus hyperproduction during asthmatic attacks (Ordonez et al., 2001). The increasing in the mucus production is related to the contribution of the infiltration of the inflammatory cells into the airway cavity. Goblet cell

metaplasia, which evolved from the nonciliated and maybe ciliated cells, is associated with the loss of the Foxa2 genes (Cardoso and Whitsett, 2008).

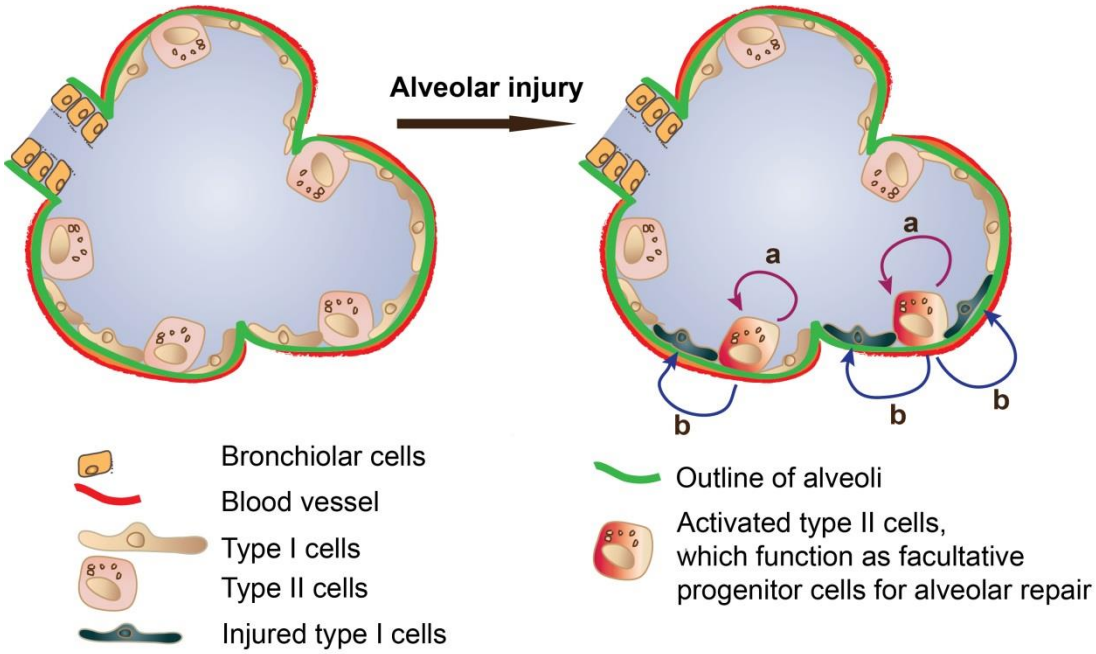


Figure 1.2 The involvement of alveolar cells during injury. Exposure to allergen causes injury will turn on the repair pathway in alveolar type II (ATII). The unfavourable condition activates the proliferation and differentiation capability of alveolar type II (ATII) cells which rapidly turn into alveolar type I cells, replacing the injured cells. (Adapted and modified from http://tigger.uic.edu/depts/mcph/lab_yuru.htm)

## 1.5 Rabbit as Animal Model of Chronic Lung Injury

Remodelling acute lung injury in medical research prospect is important in finding and understanding of the mechanism relying behind acute and chronic disease. In drug discovery study, animal has been used in eliminating the needs on manipulating human in research. The development of therapeutic strategies against complex disease requires animal as a model to mimic the aspects of respiratory diseases (Van der Velden and Snibson, 2011). In remodelling of human respiratory system, the reason in using the animal as method of the study is that the animals provide

experimental settings that allow us to understand the interaction between immune system with a functioning respiratory system.

In the requirement for choosing suitable animal to be used in the research, there are several aspects that need to be taken into consideration i.e. the ability of the animal to reproduce the principle aspects of the human lung disease, size of the animal, the accessibility to the research reagents, as well as cost in handling the animal. In order to mimic the pathophysiology of a human lung disease, animal with large size is the most suitable to be used since the complexity of the organ structure is more similar and identical to human as compared with small size animals (Matute-Bello et al., 2008). In developing the animal model of chronic lung injury, rabbit is a suitable candidate, due to its ability to develop disease from the point being sensitized with allergen for a prolonged period (Matute-Bello et al., 2008).

In a report by Keir and Page (2008), where the study used rabbit as a model for asthma and other lung diseases indicated that the allergic rabbit is sensitive to similar drugs as patients with asthma (Keir and Page, 2008). One of the drugs for asthma patients that can be used in airway disease is corticosteroid. The administration of budesonide; one type of corticosteroid in rabbit sensitized by inhalation exhibiting a marked anti-inflammatory activity, inhibits antigen-induced airway hyperresponsiveness and bronchodilator responses (Gozzard et al., 1996). Budesonide also can inhibit the early and late response of the airway hyperresponsiveness, as well as eosinophil infiltration (Keir and Page, 2008).

## 1.6 Activation of the Proliferating Genes during Repair Process

Imbalance of the cells niche after injury process will trigger the activation of several genes that regulating the maintaining of the cellular compositions. Proliferating cell nuclear antigen (PCNA) is one of the genes that actively involved during resting, proliferation, differentiation and programmed cell death (apoptosis) (Kubben et al., 1994, Kelman, 1997, Paunesku et al., 2001). PCNA is highly expressed in the cycling cells and occur during the last 5% of $G_1$-phase and the first 35% of S-phase (Kubben et al., 1994, Paunesku et al., 2001, Essers et al., 2005). The action of PCNA in cells is closely regulated by the p53 and p21 genes, which genes involving in the cell death decision either entering cell cycle arrest and undergo DNA repair or apoptosis when repair is impossible (Paunesku et al., 2001). When PCNA is driven by the p53 genes, PCNA commits the cells to cell cycle arrest and repair the DNA damage. Otherwise, when the repair is impossible, the level of functional PCNA is reduced and drives the cells into apoptosis stage (Paunesku et al., 2001). Other than that, a study by Koundrioukoff et al. (2000) showed that there was direct interaction between PCNA and CDK-2 (cyclin-dependent kinase 2), forming PCNA-Cdk2-cyclin A complex.

## 1.7 Established treatment for lung diseases

The lung serves as the second largest organs in the body and the development of lung cancer can reduce the efficacy and function of the human respiratory system in protecting the body. In preventing and treating lung diseases, some patients are subjected to undergo treatment session where they will be exposed to the various series of drugs that can combat and reduce the progression of the disease. However, there is a possibility in developing drug resistance in a cancer lineage which can be

due to the genetic changes in the normal cells where may induce changes in their biochemical properties and chemosensitivity (Nishio et al., 1999).

In treating and reducing the pain of asthma attacks, common drugs being prescript to the patients are salbuterol or salbutamol, short-acting beta agonists (SABA) inhaled bronchodilators that rapidly ease asthma symptoms (Pickholtz et al., 2011); omalizumab, given as injection which help by altering the immune system (Perkins et al., 2007); and also salmeterol, a long-acting beta agonist (LABA) that can open the airways by effectively aid in bronchodilatation for at least 12 to 18 hours (Spector, 1991). However, in using these kinds of drugs to treat the asthma attacks, there are possibilities in worsening the attacks instead of reducing it. As for SABA treatment, it can induce allergic reaction, difficulty in breathing, swelling of the mouth and for LABA, the side effects are quite similar to the SABA treatment but with increasing possibility in asthma exacerbations and risk of fatal asthma cases.

Despite the side effects of chemotherapy for cancer and drugs treatment for asthma attacks, there is a need in exploring new natural-based remedies which can be apply in treating the acute and chronic diseases. Several natural products serve as anticancer for lung cancer has been passing the clinical trial stages, such as *Catharanthus roseus* (Lu et al., 2003, Nirmala et al., 2011), *Campthotheca acuminate* (Lisa et al., 2001, Nirmala et al., 2011) , *Berberineeris* sp. (Han et al., 2010, Nirmala et al., 2011), T*abeuia avellanedae* (Nirmala et al., 2011, Higa et al., 2011) , *Vicia faba, Amoora rohituka* (Mans et al., 2000) and *Dysoxylum binectarierum* (Nirmala et al., 2011) . In treating asthma, such natural products that has been claimed to be effective against the asthma attacks are ginkgo biloba (Babayigit et al., 2009), traditional Chinese medicine; Mai Men Dong Tang, Ding

Chuan Tang and also STA-1 (Kopnina, 2012), reishi mushroom (Halpern, 2007), curcumin (Kurup and Barrios, 2008).

### 1.7.1 Virgin coconut oil (VCO) as potential anti-inflammatory and anti-asthmatic agent

Coconut (C*ocos nucifera* L.*)* is commonly growth at tropical country, especially in Asia region. A coconut consists of husk, hard shell, copra and water, which dominantly occupied the fruit. A mature coconut often harvested and served as raw materials; copra for coconut oil production, hard shell to be used in charcoal industries and husk can be used to make coir (Figure 1.3). Commonly used part in the coconut is the endoderm or copra. Copra, with high oil content with about 65-75% oil (DebMandal and Mandal, 2011) is usually used in both food and non-food industries, such as soap, lotion or margarine production. The mass production of the by-product requires large scale of the coconut oil supply and Malaysia produce about 52.17% of coconut oil in 2013 to fulfil the requirement for the industries (United States Department of Agriculture). Coconut oil can be extracted from the copra meat through various methods, such as traditional heat-expelling method and solvent extraction method (Nevin and Rajamohan, 2009). Both methods extremely affect the nutritional content of the coconut oil due to the heat applied during processing methods. Moreover, the coconut oil or also known as Refined, Bleached and Deodorized (RBD) oil turn out to be not as beneficial as virgin coconut oil (VCO) since the coconut fat is deodorized at a maximum temperature of 220°C for 4 hours at a pressure between 3-5 bar and need to be purified prior human consumption due to the unsanitary drying processing of the copra.

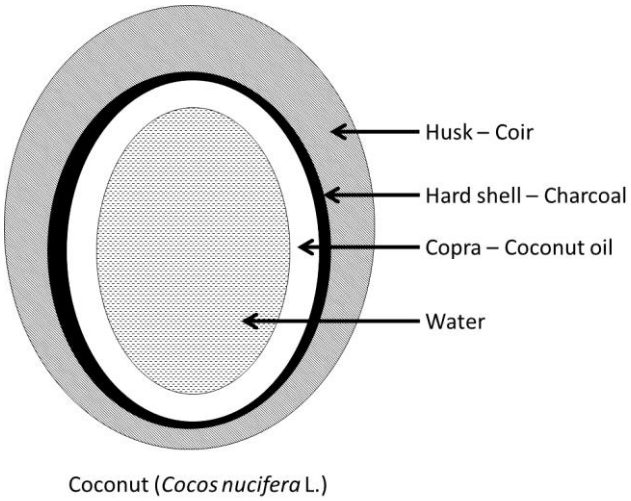


Figure 1.3 Cross-section of a coconut (*Coconut nucifera* L.). The mature coconut often harvested to be used in industries production of coir (husk), charcoal (hard shell), and coconut oil (copra).

Currently, the trend in consuming natural products has been greatly impact the growth of interest on VCO. VCO is widely used around the world, especially in tropical region such as South and Central America, Africa, Indian subcontinent and commonly used in Asia, especially in Malaysia, Thailand and Philippines (Verallo-Rowell et al., 2008, Marina et al., 2009). In contrast with RBD coconut oil, the VCO is obtained directly from coconut milk by wet process under controlled temperature, which can prevent the loss of biologically active minor components like vitamins and polyphenols (Zakaria et al., 2011a, Zakaria et al., 2011b). VCO is one of the natural products that rich in antioxidants which high in phenolic acid (ferulic acid, and p-coumaric acid) (Liau et al., 2011, Marina et al., 2009, Nevin and Rajamohan, 2009, Zakaria et al., 2011a). According to APCC (Asian and Pacific Coconut Community) standard for VCO, most detectable free fatty acids is lauric acid (C12:0), with 45-56% of total content, followed by ranges of fatty acids with 6-18 carbon chains.

Free fatty acids available in this natural oil has been studied their beneficial effects on many aspects of health diseases. Various studies has been done by scientists

worldwide with some reported that VCO exhibits anti-bacteria (Nevin and Rajamohan, 2010), anti-viruses, anti-HIV, anti-inflammatory properties (Zakaria et al., 2011b), anti-diabetes (Nevin and Rajamohan, 2004, Liau et al., 2011), and has been extensively used as topical application in treating skin disorders (Agero and Verallo-Rowell, 2004, Verallo-Rowell et al., 2008, Nevin and Rajamohan, 2009). Study by Badlishah S et.al (2013) showed that VCO able to prevent blood pressure elevation and improves endothelial function in rats (Nurul-Iman et al., 2013). The efficacy and safety of the virgin coconut oil in reducing visceral adiposity also has proved that the VCO is efficacious as weight reducing and safe for human consumption (Liau et al., 2011). A study on toxicity of the VCO has been done by a group of researchers from USM. The study tested the VCO on rat model of acute toxicity test, where they found that high dosage (2000mg/kg) of oral consumption of VCO give no visible signs of toxicity and mortality (Ahamed et al., 2011). The available study on treating cancer using VCO has been done by Calderon et.al (2009) and Law et al. (2014) where they investigated the effect of VCO on breast cancer progression both *in vitro* and *in vivo* (Law et al., 2014, Calderon et al., 2009). However, to date, such study in assessing the effectiveness of VCO in alleviating asthmatic cellular response has never been reported by other researchers. In order to fill in the gap between traditional claim and scientific-based evident, this study was aimed to evaluate the efficacy of the VCO either reducing the asthma pathogenesis or preventing the onset of asthma.

## 1.8 Objectives of the study

### 1.8.1 General objective

The main objective of this study was to investigate the effects of aerosolisation of VCO on ovalbumin-induced (Ova) airway injury, which involved the inflammatory cell infiltration, airway remodelling and mucus overproduction.

### 1.8.2 Specific objectives

The specific objectives of this study;

a) To investigate the effect of VCO treatment on the pathophysiological changes of the airway structures (epithelium, mucosa and submucosa) in response to Ova-induced airway injury.
b) To investigate the effect of VCO treatment on inflammatory response (eosinophil, basophils, neutrophils, lymphocytes and monocyte) of the airways following Ova-induced airway injury.
c) To study the effect of VCO treatment on mucus-producing goblet cells following Ova-induced airway injury.
d) To study the effect of VCO treatment on the proliferation of the airway epithelial cells following Ova-induced airway injury.
e) To screen the anti-inflammatory related active compounds of the crude extract of VCO using GC-MS profiling method.

## 1.9 Research Hypothesis

The inhalation of VCO using portable nebulizer is able to reduce the infiltration of inflammatory cells, which help in reconstructing the airway structure, reduce the goblet cell hyperplasia and the proliferation activity of the airway epithelial cells following injury in animal model of asthma.

## 1.10 Relevance of the Study

This study was proposed to elucidate the effects of the VCO in alleviating the pathogenesis of asthma and/or preventing the onset of asthma-related cellular response. The availability of the commercialised drug-based products for asthma treatment have no doubt give significant effect on asthma pathogenesis, still with long list of side effects. The traditional use of VCO have been claimed to be effective in various diseases including asthma, but no scientific evident has yet available to demonstrate its efficacy in treating asthma. This study was aimed to provide scientific evidence in current therapy for asthma by using aerosol-based VCO treatment which can provide a better delivery route of the VCO into injured lungs compare to the common use of VCO (oral consumption and tropical application). This study was also aimed to provide an alternative way of treating asthma patients using aerosol-based VCO treatment, which mimicking drug delivery route of the current drug therapy for asthma (SABA or LABA) patient using portable nebulizer.

# CHAPTER 2

# MATERIAL AND METHOD

## 2.1 Overview of the study

The study involved development of the animal model of chronic lung disease (asthma) by exposing to the i.p Ova and alum and Ova-inhalation. The animal were grouped into five different groups, which were naïve (untreated, normal animal), i.p. OVA (receive only i.p. Ova treatment), Ova-inhalation (receive i.p. Ova and inhalation of Ova), VCO as rescue agent (receive VCO as rescue treatment) and VCO as preventive agent (receive VCO as preventive treatment). After that, the samples (blood, BAL fluid and lung) were collected and proceed with sample analyses (Figure 2.1).

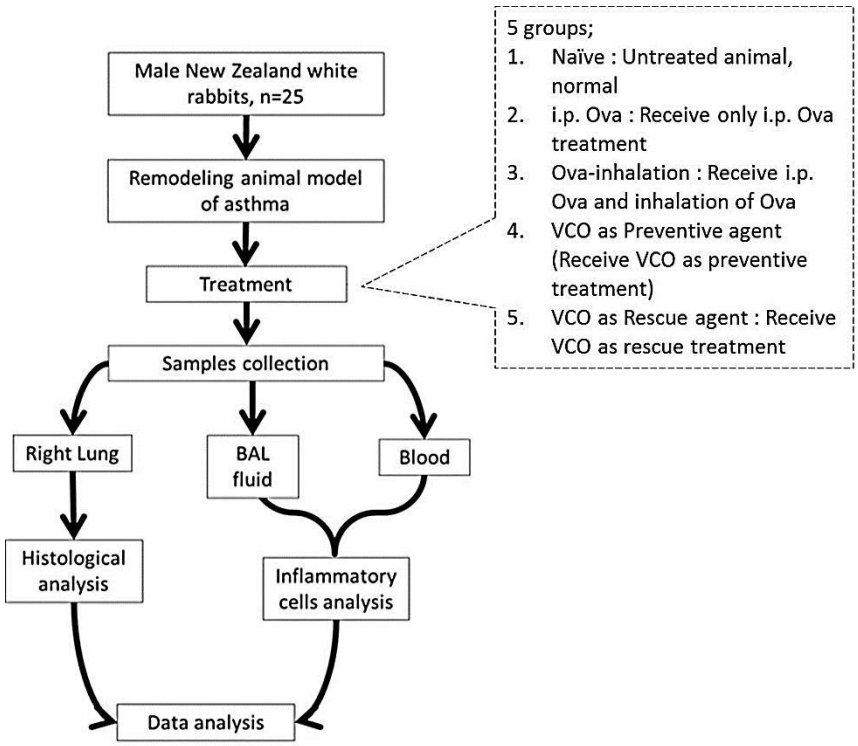


**Figure 2.1** The overview for the current study. The *in vivo* study was aimed to observe the effectiveness of VCO in reducing the injury level of ovalbumin-induced animal model.

## 2.2 The effect of VCO on the airway of rabbit model for asthma

### 2.2.1 Source of VCO

The VCO used in this study was purchased from Nutrifera Sdn. Bhd. (Kelantan Biotech Corporation, MY).

### 2.2.2 Animal model

Adult male New Zealand white rabbits (n=25), aged 4-6 months and weighted 2.60 ± 0.38 kg, were used in this study. The ethical approval were obtained from *The Animal Research Ethics Committee of USM (JEHUSM)* (USM/Animal Ethics Approval/2012/ (77) (379) (Appendix 1). The rabbits were maintained in ventilated room and fed daily with water and pellets.

The rabbits were divided into five independent groups (n=5 for each group) where two groups received allergen exposure and another two groups received VCO-inhalation treatments and one serve as control (untreated). Two groups that received allergen exposure were served as injury for; 1. intraperitoneal injection (i.p.) Ova only and 2. Ova-inhalation (i.p. Ova and Ova-inhalation). These two groups were combined together and later known as Ova-induced injury group, and another two served as treatment groups, which later known as the preventive and rescue groups. In Ova-induced injury group, the rabbits were sensitized with ovalbumin (i.p. Ova) twice, containing 1ml Ova (0.1 mg/ml ovalbumin, (Ova), grade V) (Sigma Aldrich, US) and 1 ml Alum (10 mg/ml aluminium hydroxide (alum)) (Thermo Fisher Scientific, US) on day 1 and 14 respectively (Figure 2.2). Twenty-four hours following second i.p. Ova injection, rabbits in i.p. Ova control group were euthanized. In Ova control group, the rabbits were aerosolized with 5 ml Ova (10 mg/ml Ova) for three subsequent days (days 28, 29, and 30) using nebulizer

(MABISMist handheld Ultrasonic Nebulizer, US) for 20 mins and was euthanized on day 31.

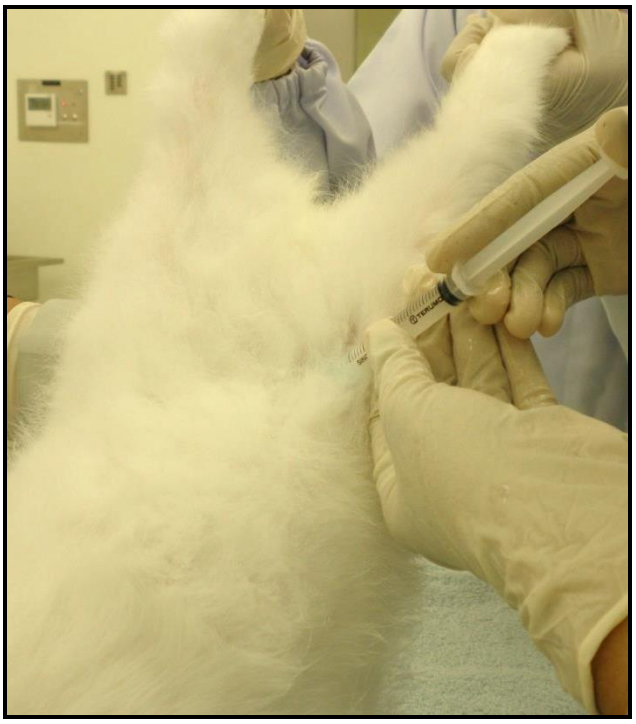

**Figure 2.2** Intraperitoneal (i.p.) injection of ovalbumin on day 1 and 14 during asthmatic sensitization stage on the rabbits.

As for the treatment groups, following the second i.p. Ova injection, the rabbits in both rescue and preventive groups were given repeated inhalation of 5 ml of 100% VCO (Nutrifera, MY) by using nebulizer for 20 mins for five days (± 2 ml/20 mins), which was from the day 23 until day 27. Following VCO-inhalation, the rabbits from rescue group were euthanized on day 28. However, in preventive group, following five days of VCO inhalation (day 23 until day 27), the rabbits were given 5 ml of Ova (10 mg/ml) inhalation for another three days, which was on day 28 until day 30. The rabbits were then euthanized on day 31$^{st}$. Figure 2.3 represents the timepoint for each group that were subjected to the development of asthma and treated with the VCO.

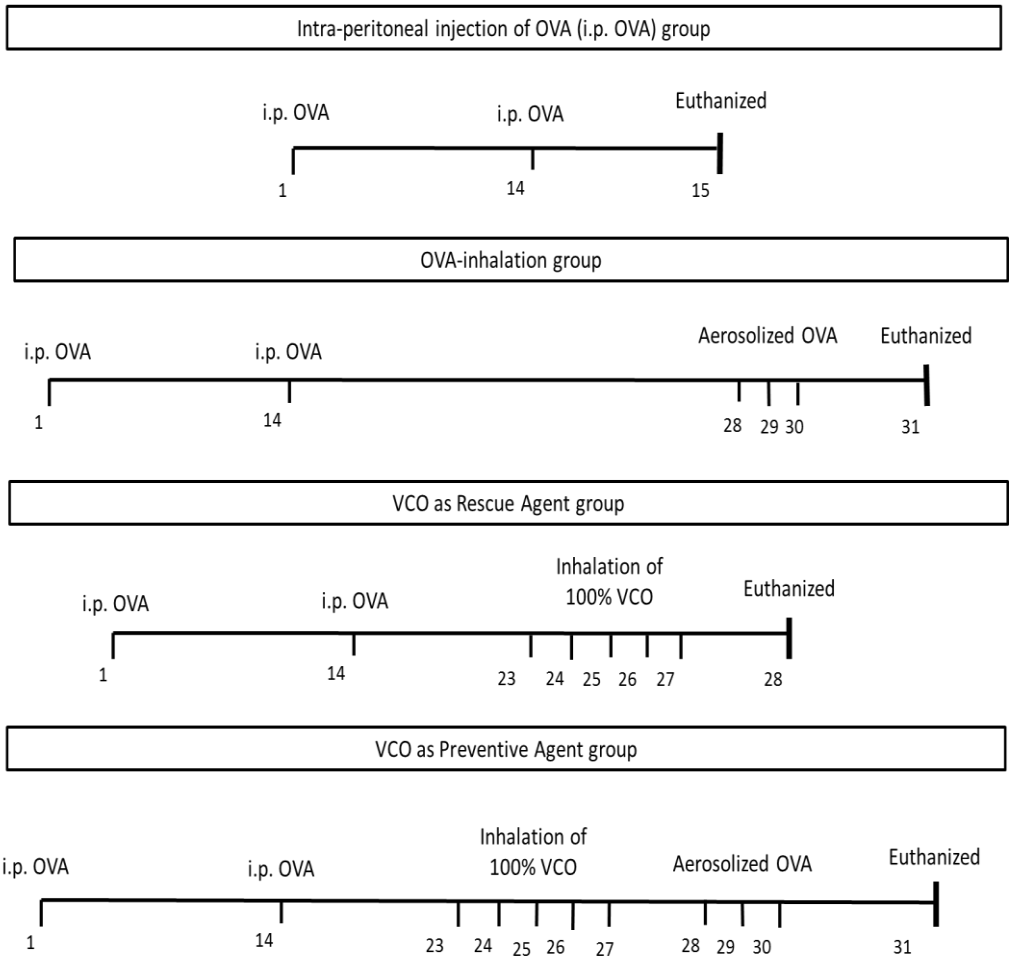


**Figure 2.3** Timeline for animal groups. Control group for Ova-inhalation; Control group for intraperitoneal injection of Ova (i.p. Ova); VCO as rescue agent; and VCO as preventive agent. Adapted and modified from (Kumar et al., 2008, Olmez et al., 2009, Lee et al., 2012, Kamaruzaman et al., 2014).

### 2.2.3 Aerosolisation of VCO into the lung of the asthma-induced rabbits

Ovalbumin-induced rabbits were treated via inhalation of VCO using nebulizer (Figure 2.4). The rabbits were given 5ml Ova (10mg/ml Ova in PBS) using the MabisMist™ Ultrasonic Nebulizer (MABIS Healthcare INC, US) for 20 mins. The MabisMist™ Ultrasonic Nebulizer is portable ultrasonic nebulizer that is commonly used for asthma treatment.


**Figure 2.4** The aerosolisation process of VCO into the lungs of the rabbits using MabisMist™ Ultrasonic Nebulizer. The process takes 20 mins for a cycle to be completed.

### 2.2.4 Blood collection for whole blood count (WBC)

In this study, the blood was collected on day 28 or 31, prior to post-mortem of the rabbits. The rabbits were first anesthetised using ketamine (35 mg/kg) (Ilium, NSW, Australia) and ilium xylazyl (4 mg/kg) (Ilium, NSW, Australia) via intramuscular (i.m.) injection. Once the rabbits were unconscious, 5ml syringe was punctured into the rabbits' heart and the 3 ml blood was drawn out (Figure 2.5). The blood was then transferred into 3ml blood collection tube and was kept in -20˚C refrigerator. The whole blood count was performed using automated blood counter (Cell-Dyn ® 3700, Abbott Diagnostic, US). Following the blood collection, the rabbits were euthanized

with overdosed pentobarbital (1 ml/kg) (Dolethal, Lure Cedex, France) and subjected for post mortem.

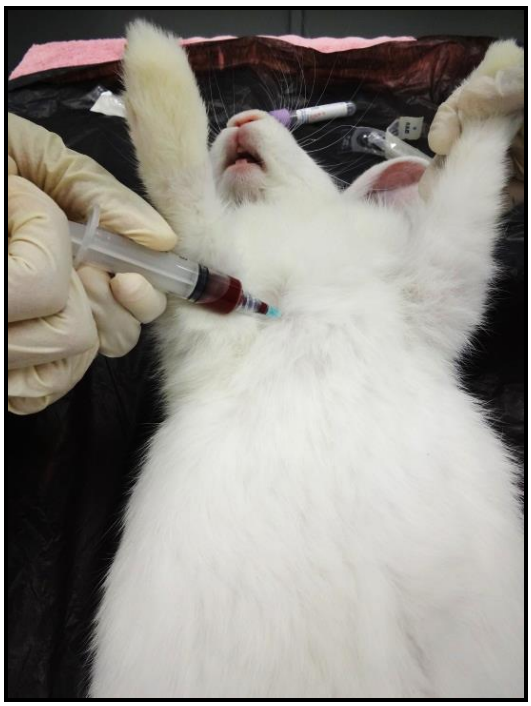

**Figure 2.5** The syringe was directly injected into the heart and the blood was drawn out and collected into EDTA tube. The tubes containing blood were kept at 4°C until usage.

### 2.2.5 Collection of bronchioalveolar lavage (BAL) fluid

The rabbits were first euthanized to cut open the chest to expose the trachea. The proximal area of the lung was cut out and BAL fluid from each rabbit was collected by lavaging the lung via trachea with 3 ml of PBS, twice (Figure 2.6). The collected BAL fluid was then centrifuge at 400x g for 5 min (4 °C). The supernatant was transferred into new 1.5 ml collection tube and stored at -70°C. The pellets were then re-suspended in PBS and proceed with standard cell counting to be used in the cytospin method and whole white blood cell count.

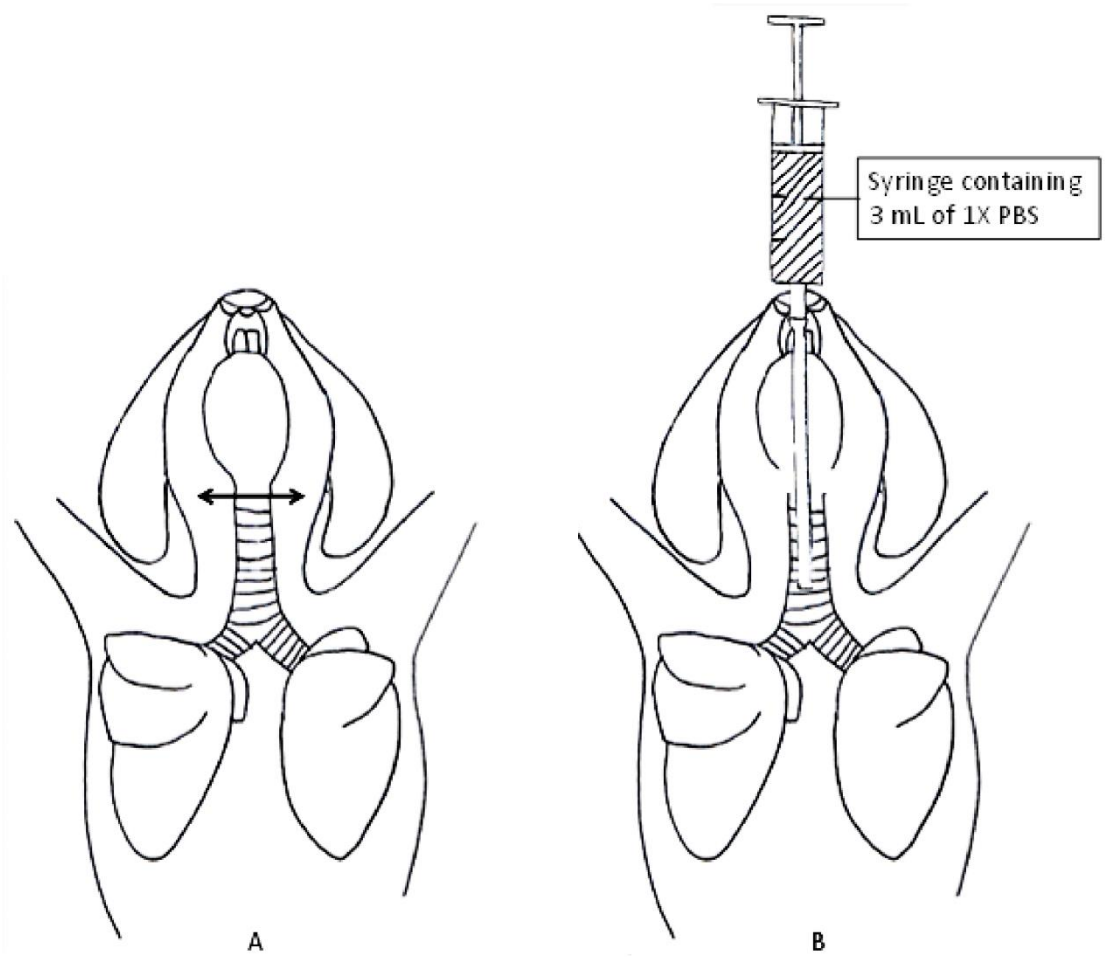


**Figure 2.6** Cross section of the rabbit during BAL fluid collection. The trachea was cut at epiglottis area (A). The syringe containing 3 mL of 1X PBS was inserted through the opening area. The BAL fluid was then collected in 1.5 mL tube.

### 2.2.6 Preparation for cytospun of BAL fluid

For this purpose, the numbers of cells were adjusted to $1x10^5$ cells/ml. About 100 µl of BAL fluid cell suspension was transferred into cytofunnel attached with cytoslide. The cytofunnel containing cells was then placed into cytospin machine (Thermo Scientific Shandon Cytospin® 4 Cytocentrifuge, US). The cytospin was closed and the samples were spun at 1500 rpm to avoid cell death for 5 mins. After the cytospin was stopped, the filter was carefully removed from cytofunnel, without contacting the cell smears on the slides. The slides were left air dried in a flat layer on the slide

prior to standard Wreight-Giemsa staining process. Identification process of the inflammatory cell and cell scoring was done by a pathologist.

### 2.2.7 Whole blood count for inflammatory cell

The inflammatory cell count was done in order to observe the pattern of the inflammatory cell infiltration into the airway structure following the Ova-induced injury. The pellet from BAL fluid was diluted into 1x PBS with ratio 1:10. For counting purpose, 100 µl of BAL fluid was injected into an automated blood counter (Cell Dyn 3700 SL, Abbott Labortories, US). The inflammatory cell counting was expressed as inflammatory cell percentage (%).

### 2.2.8 Collection, processing and sectioning the lung tissue

The lung and trachea of the rabbits were collected right after BAL fluid collected. The lung was divided into two parts, where right lung was fixed in 10% (v/v) formalin for histological study meanwhile left lung was kept for future study. Twenty-four hours after fixation in 10% formalin, the right lung was cut in cross section manner, into small blocks and placed in histological cassette (Figure 2.7). The histological cassettes containing tissues were then transferred into an automated Excelsior ES Tissue Processor (Thermo Fisher Scientific, US) and left overnight. In this stage, the tissues were subjected to dehydration, clearing and infiltration process. Once the tissues were processed, the tissue blocks were removed from the machine and transferred into the tissue embedding machine (Shandon Histocentre 3 Embedding Centre, US), where the tissue blocks were placed into embedding cassettes and filled with paraffin. The paraffin-filled embedding cassettes were left on cold platform until hardened and subjected to sectioning into 5 micron thickness using a microtome (Accu-Cut® SRM™ 200, Sakura®, US). The tissue ribbons were

left air-dried on normal slides (haematoxylin & eosin (H&E) staining, alcian blue-periodic Acid-Schiff (AB-PAS) staining) and polysine-coated slides (immunohistochemical (IHC) staining) process.

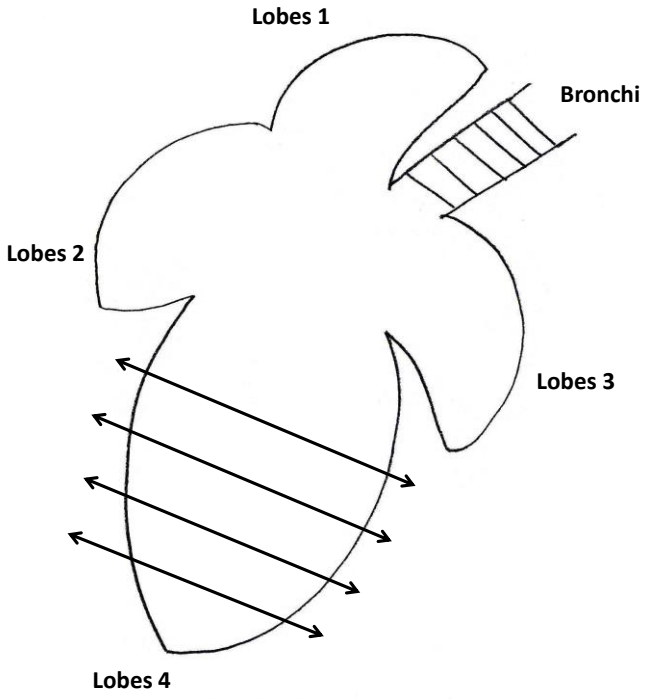


**Figure 2.7** Diagram of the lung of the rabbit. All lobes (lobes 1, 2, 3 and 4) were cut in cross section manner (double-arrowhead), so that the internal airway structure of the lung can be observed and examined under microscope for further analysis.

### 2.2.9 Heamatoxylin & Eosin (H&E) staining

Lung sections were subjected to the H&E staining using standard protocol. The lung sections were deparaffinised in xylene twice for 2 min and dehydrated in gradient series of ethanol (100%, 90%, 80%, 70% and 50%) for 2 min. The slides were then rinsed under running tap water for 2 min. After that, the slides were immersed in Harris-haematoxylin solution (Accustain®, Sigma Aldrich, Germany) for 11 min and rinsed with running tap water until blue colour dye of Harris-haematoxylin