Investigating the mechanisms underlying stroke outcomes in chronic obstructive pulmonary disease

A thesis submitted in fulfillment of the requirements for the degree of Master of Science

Victoria Austin
BBiomed (Hons)

School of Health and Biomedical Sciences
College of Science, Engineering and Health
RMIT University

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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# Table of Contents

DECLARATION  

I  

ACKNOWLEDGEMENTS  

II  

TABLE OF CONTENTS  

III  

LIST OF FIGURES AND TABLES  

VII  

COMMON ABBREVIATIONS  

IX  

ABSTRACT  

1  

CHAPTER 1 GENERAL INTRODUCTION  

2  

1.1 Chronic obstructive pulmonary disease (COPD)  

3  

1.2 Overview of COPD pathophysiology  

4  

1.3 Acute Exacerbations of COPD  

5  

1.4 Oxidative stress in COPD and AECOPD  

6  

1.5. Evidence of systemic inflammation and oxidative stress in COPD  

8  

1.6. COPD and cardiovascular disease  

9  

1.7 Overview of stroke pathophysiology  

10  

1.8 Evidence linking lung function, COPD and stroke  

13
1.8.1 Link between poor lung function and risk of cerebral events 13
1.8.2 COPD and risk of clinical stroke 14

1.9 Potential mechanisms linking COPD and stroke risk and severity 14
1.9.1 Contribution of shared risk factors 14
1.9.2 Association with traditional stroke risk factors 16
1.9.3. COPD-specific systemic inflammation and oxidative stress 17

1.10 COPD and stroke outcomes 21
1.11 Lung injury after stroke 21
1.12 Aims of this thesis 22

CHAPTER 2 GENERAL METHODS 24

2.0 Methods 25
2.1 Animals and ethics 25
2.2 Comparison of stroke outcome in two mouse strains: BALB/c and C57BL/6 25
2.2.1 Focal cerebral ischaemia and reperfusion via transient middle cerebral artery occlusion (tMCAO) 26
2.2.2 Neurological scoring & Functional tests 28
2.2.3 Quantification of infarct and oedema volumes 29

2.3 Effect of stroke on lung inflammation and lung injury 29
2.3.1 Bronchoalveolar lavage and differential cell counts 30
2.4 Effect of cigarette smoke exposure on stroke outcome

2.4.1 RNA extraction

2.5 Data and statistical analysis

CHAPTER 3 COMPARISON OF STROKE OUTCOME IN TWO MOUSE STRAINS:
BALB/C AND C57BL/6

3.1 Introduction

3.2 Materials and methods

3.2.1 Animals and ethics

3.2.2 Experimental protocols

3.3 Results

3.3.1 Degree of hypoperfusion after stroke

3.3.2 Stroke outcomes in C57BL/6 and BALB/c mice

3.3.3 Cerebral infarct and oedema volumes in C57BL/6 and BALB/c mice

3.4 Discussion

CHAPTER 4 EFFECT OF STROKE ON THE LUNG

4.1 Introduction

4.2 Materials and methods

4.2.1 Animals and ethics

4.2.2 Experimental protocols
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3 Results</td>
<td>46</td>
</tr>
<tr>
<td>4.3.1 Degree of hypoperfusion after stroke</td>
<td>46</td>
</tr>
<tr>
<td>4.3.2 Functional and neurological outcomes of stroke</td>
<td>47</td>
</tr>
<tr>
<td>4.3.3 Effect of stroke on lung inflammation</td>
<td>50</td>
</tr>
<tr>
<td>4.4 Discussion</td>
<td>53</td>
</tr>
</tbody>
</table>

CHAPTER 5 EFFECT OF CIGARETTE SMOKE EXPOSURE ON STROKE OUTCOME

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Introduction</td>
<td>58</td>
</tr>
<tr>
<td>5.2 Materials and methods</td>
<td>59</td>
</tr>
<tr>
<td>5.2.1 Animals and ethics</td>
<td>59</td>
</tr>
<tr>
<td>5.2.1 Experimental Protocols</td>
<td>59</td>
</tr>
<tr>
<td>5.3 Results</td>
<td>60</td>
</tr>
<tr>
<td>5.3.1 Effect of cigarette smoke exposure on body weight and lung</td>
<td>60</td>
</tr>
<tr>
<td>inflammation</td>
<td></td>
</tr>
<tr>
<td>5.3.2 Degree of hypoperfusion following stroke</td>
<td>65</td>
</tr>
<tr>
<td>5.3.3 Effect of cigarette smoke exposure on stroke outcomes</td>
<td>67</td>
</tr>
<tr>
<td>5.3.4 Effect of cigarette smoke exposure on infarct and oedema</td>
<td>69</td>
</tr>
<tr>
<td>5.4 Discussion</td>
<td>70</td>
</tr>
</tbody>
</table>

CHAPTER 6 CONCLUSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>REFERENCES</td>
<td>78</td>
</tr>
</tbody>
</table>
List of Figures and Tables

**Figure 1.1:** Possible mechanisms underlying COPD and stroke risk

**Figure 2.1:** Path of filament and position of Laser Doppler probe in tMCAO model.

**Figure 2.2:** Expected changes in regional cerebral blood flow (rCBF) during tMCAO.

**Figure 3.1:** Regional cerebral blood flow during stroke surgery and during reperfusion in BALB/c and C57BL/6 mice.

**Figure 3.2:** Hanging wire and neurological scores after stroke surgery with an ischaemic period of 30 minutes in C57BL/6 mice and BALB/c mice, and in C57BL/6 mice after a sham procedure.

**Figure 3.3:** Representative thionin-stained coronal brain sections of BALB/c mice at 24 h post-stroke.

**Figure 3.4:** Cerebral infarct and oedema volumes at 24 h after stroke procedure with ischaemic periods of 30 min and 50 min in BALB/c and C57BL/6 mice.

**Figure 4.1:** Regional cerebral blood flow during stroke surgery and during reperfusion in C57BL/6 mice.

**Figure 4.2:** Neurological deficit scores 6 h and 24 h post-stroke or sham surgery, and hanging wire tests 24 h post-stroke surgery in C57BL/6 mice.

**Figure 4.3:** Infarct and oedema volumes at 24 h post-stroke in C57BL/6 mice.

**Figure 4.4:** Inflammatory cells in bronchoalveolar lavage fluid (BALF) at 6 h and 24 h post-stroke in C57BL/6 mice.

**Figure 4.5:** mRNA expression of proinflammatory genes in whole lung tissue as detected by qPCR, 6 h post-stroke in C57BL/6 mice.
**Figure 4.6:** mRNA expression of proinflammatory genes in whole lung tissue as detected by qPCR, 24 h post-stroke in C57BL/6 mice.

**Figure 5.1:** Effect of 2 weeks cigarette smoke exposure on body weight.

**Figure 5.2:** Effect of 8 weeks cigarette smoke exposure on body weight.

**Figure 5.3:** Effect of 12 week smoke exposure on body weight.

**Figure 5.4:** Effect of 2 weeks cigarette smoke exposure on inflammatory cells in bronchoalveolar lavage fluid (BALF).

**Figure 5.5:** Effect of 8 weeks of cigarette smoke exposure on inflammatory cell counts in bronchoalveolar lavage fluid (BALF).

**Figure 5.6:** Effect of 12 weeks cigarette smoke exposure on inflammatory cell counts in bronchoalveolar lavage fluid (BALF).

**Figure 5.7:** Regional cerebral blood flow during stroke surgery and during reperfusion, following 2 weeks of cigarette smoke or sham smoke exposure.

**Figure 5.8:** Regional cerebral blood flow during stroke surgery and during reperfusion, following 8 weeks of cigarette smoke or sham smoke exposure.

**Figure 5.9:** Regional cerebral blood flow during stroke surgery and during reperfusion, following 12 weeks of cigarette smoke or sham smoke exposure.

**Figure 5.10:** Effect of 2, 8 or 12 weeks of cigarette smoke exposure on neurological deficit and functional hanging wire scores at 24 h after stroke.

**Figure 5.11:** Effect of 2, 8 or 12 weeks of cigarette smoke exposure on cerebral infarct and oedema volumes at 24 h after stroke.
## Common Abbreviations

A list of common abbreviations used across all chapters is provided below.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AECOPD</td>
<td>Acute exacerbations of chronic obstructive pulmonary disease</td>
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<td>ALI</td>
<td>Acute lung injury</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>CIAD</td>
<td>Chronic inflammatory airway disease</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CS</td>
<td>Cigarette smoke</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IL-8</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTB₄</td>
<td>Leukotriene B4</td>
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<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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All units used through this thesis comply with the International System of Units (SI) including units officially accepted for use with the SI.

<table>
<thead>
<tr>
<th>Symbol</th>
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</tr>
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<tr>
<td>h</td>
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Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive, incurable lung disease with various systemic comorbidities. The COPD population has a higher risk of cardiovascular comorbidities, including stroke. There is some evidence to suggest worse outcomes after stroke in the COPD population. It has also been shown that pulmonary complications frequently occur after stroke. Therefore, the primary aims of this thesis were to investigate whether COPD impacts on stroke outcomes, and inversely, whether stroke causes acute lung injury in mice. This thesis has systematically examined (i) strain-dependant differences of BALB/c and C57BL/6 mice in response to an experimental model of stroke, transient middle cerebral artery occlusion (tMCAO), (ii) changes in markers of lung injury in mice following experimental stroke and (iii) changes in stroke outcomes in C57BL/6 mice exposed acutely and chronically to cigarette smoke (preclinical mouse model of COPD). There was no observable difference in stroke outcomes between C57BL/6 and BALB/c mice, however the BALB/c mice had a high mortality rate and thus were not used for ongoing studies. Lung inflammation, as measured through inflammatory cell counts in bronchoalveolar lavage fluid and proinflammatory gene expression in lung tissue, was elevated 6 h after ischaemic stroke. Further work needs to be done to characterise the lung inflammatory response to experimental ischaemic stroke. Chronic cigarette smoke exposure did not worsen stroke outcomes in this study. The period of smoke used in this study may have been insufficient to induce systemic changes believed to play a key role in worsened stroke outcomes.
Chapter 1

General Introduction
1.1 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a major incurable global health burden and is currently the third largest cause of death in the world (1-3). Much of the disease burden and health care utilisation in COPD is associated with the management of its comorbidities and infectious (viral and bacterial) exacerbations, known as acute exacerbations of COPD (AECOPD). In the United States alone, the medical costs attributed to COPD in 2010 were estimated to be in excess of $32 billion (4). Comorbidities, defined as other chronic medical conditions, in particular cardiovascular disease markedly impact on disease morbidity, progression and mortality. Indeed, it is estimated that between 30 and 50% of COPD-related deaths are due to a cardiovascular comorbidity such as coronary artery disease, hypertension and diabetes (5-7). In addition, patients with COPD are at increased risk for stroke and this is even higher in the weeks following an AECOPD (8, 9). Moreover, there is some evidence to suggest that COPD is associated not only with an increased risk of stroke, but worse outcomes following stroke (10, 11). Importantly, COPD is an independent predictor of mortality after stroke (10).

The mechanisms and mediators underlying COPD and its comorbidities, in particular elevated stroke risk and severity, are poorly understood. However, there is compelling evidence to suggest that increased lung inflammation oxidative stress and the “spill over” into the systemic circulation and play an important role in the pathophysiology of COPD and its comorbidities such as stroke. Therefore, while there are currently no effective therapies for reversing the lung pathology that is characteristic of COPD (12), targeting oxidative stress and lung/systemic inflammation could prove to be an effective way to treat the
comorbidities associated with COPD and hence improve survival and quality of life in these patients.

1.2 Overview of COPD pathophysiology

COPD is a disease characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of lungs to noxious particles and gases (13, 14). Cigarette smoking is the major cause of COPD and accounts for more than 95% of cases in industrialized countries (15), but other environmental pollutants are important causes in developing countries (16). COPD encompasses chronic obstructive bronchiolitis with fibrosis and obstruction of small airways, and emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways. Most patients with COPD have all three pathologic conditions (chronic obstructive bronchiolitis, emphysema and mucus plugging), but the relative extent of emphysema and obstructive bronchiolitis within individual patients can vary (17).

It is well established that a number of inflammatory cell types are involved in the pathophysiology of COPD including macrophages, neutrophils and T-cells (reviewed in (17-20)). These cells release a variety of mediators (e.g. tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), reactive oxygen species (ROS), leukotriene B4 (LTB₄), interleukin-8 (IL-8), granulocyte macrophage-colony stimulating factor (GM-CSF), elastolytic enzymes such as neutrophil elastase and matrix metalloproteinases) in response to cigarette smoke which orchestrate and perpetuate the inflammatory response
in COPD (reviewed in (17-20)). In addition to an increase in the number of macrophages and neutrophils, these cells appear to have an impaired phagocytic function, resulting in impairment in clearance of apoptotic cells and potentially contributing to the chronic inflammatory state in the lungs (17). The above events promote further inflammation creating a feedback loop that leads to chronic inflammation (17). Chronic inflammation induces repeated cycles of injury and repair that result in structural remodelling of the airway walls (collagen deposition, mucus hypersecretion), destruction of the parenchyma and alveolar walls and hence alveolar enlargement and emphysema (17). Once induced, the patients’ condition progressively deteriorates with worsening inflammation, emphysema, declining lung function and increased breathlessness (12). Importantly, the mechanisms and mediators that drive the induction and progression of chronic inflammation, emphysema and altered lung function are not well understood, and this has severely hampered the development of effective treatments for COPD. In addition, current treatments have limited efficacy in inhibiting chronic inflammation, do not reverse the pathology of disease and fail to modify the factors that initiate and drive the long-term progression of disease (20). Therefore, there is a clear and demonstrated need for new therapies that can prevent the induction and progression of COPD.

1.3 Acute Exacerbations of COPD

An acute exacerbation of COPD (AECOPD) is defined as “a sustained worsening of the patient’s condition, from the stable state and beyond normal day to day variation, which is acute in onset and necessitates change in regular medication in a patient with underlying COPD” (21). Exacerbations are a
common occurrence in COPD patients and contribute mainly to morbidity, death and health-related quality of life (21). AECOPD is a major cause of avoidable hospital admissions and often due to viral and bacterial infections with 40%-60% attributed to viral infections alone (21). The majority of these infections are due to respiratory syncytial virus (22%), influenza A (25%) and picornavirus (36%), with influenza having the potential to be more problematic due to the likelihood of an epidemic (21-23). Respiratory viruses produce longer and more severe exacerbations and have a major impact on health care utilization (23, 24). Currently, bronchodilator combinations modestly reduce the risk of exacerbation by about 30% and are even less effective at preventing severe exacerbations that result in hospitalization (21).

The understanding of the cellular and molecular mechanisms underlying AECOPD are limited, but there is an increase in neutrophils and concentrations of IL-6, IL-8, TNF-α and LTB₄ in sputum during an exacerbation (25, 26) and patients who have frequent exacerbations have higher levels of IL-6 and lower concentrations of secretory leukocyte protease inhibitor (SLPI), even when COPD is stable (27, 28). There is also an increase in the activation of NFκB in alveolar macrophages during exacerbations of COPD (29), which is indicative of an inflammatory environment.

1.4 Oxidative stress in COPD and AECOPD

There is now extensive evidence to show that oxidative stress plays an important role in COPD given the increased oxidant burden in smokers (30, 31). Oxidative stress is initiated by cigarette smoke which has more than $10^{14}$ relatively long-lived oxidants/free radicals per puff (32). These oxidants give
rise to secondary reactive oxygen species (ROS) by inflammatory and epithelial cells within the lung as part of an inflammatory-immune response towards a pathogen or irritant. Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) on macrophages, neutrophils and epithelium generates superoxide radicals (O$_2^*$), which can then either react with nitric oxide (NO) to form highly reactive peroxynitrite molecules (ONOO$^-$) or alternatively be rapidly converted to damaging hydrogen peroxide (H$_2$O$_2$) under the influence of superoxide dismutase (SOD) (33-36). This in turn can result in the non-enzymatic production of damaging hydroxide radical (•OH) from H$_2$O$_2$ in the presence of Fe$^{2+}$. Polymorphisms in extracellular SOD have been associated with reduced lung function and susceptibility to COPD (37). Glutathione peroxidases (Gpx) and catalase serve to catalyse toxic H$_2$O$_2$ into water and oxygen. The ROS O$_2^*$, ONOO$, H_2O_2$ and ‘OH then trigger extensive inflammation, DNA damage, protein denaturation and lipid peroxidation (32). Consequently, smokers and patients with COPD have higher levels of exhaled ROS than non-smokers, and these levels are further increased during exacerbations (38, 39).

We have shown that loss of the anti-oxidant enzyme Gpx-1 resulted in augmented cigarette smoke-induced lung inflammation compared to sham-exposed wild type mice and that synthetic repletion of Gpx activity with ebselen reduced cigarette smoke-induced lung inflammation and damage (40).

Alveolar macrophages obtained by BAL from the lungs of smokers are primed to release greater amounts of ROS compared with those obtained from non-smokers (41). Exposure to cigarette smoke in vitro has also been shown to increase the oxidative metabolism of alveolar macrophages (42). Subpopulations of alveolar macrophages with a higher granular density appear
to be more prevalent in the lungs of smokers and are responsible for the increased $O_2^{**}$ production associated with macrophages from smokers (42, 43). The generation of ROS in epithelial lining fluid may be further enhanced by the presence of increased amounts of free iron in the pulmonary airspaces in smokers (44). This is relevant to COPD since the intracellular iron content of alveolar macrophages is increased in cigarette smokers (45). In addition, macrophages obtained from smokers release more free iron in vitro than those from non-smokers (46).

1.5. Evidence of systemic inflammation and oxidative stress in COPD

In addition to lung inflammation, a state of chronic systemic inflammation is observed in COPD (47). Studies have shown increases in the serum levels of C-reactive protein (CRP), fibrinogen, serum amyloid A and different pro-inflammatory cytokines including TNF-α, IL-6 and IL-8 in COPD patients (48-50). Importantly, these markers of systemic inflammation are elevated even further during AECOPD (51). The origin of this systemic inflammation remains unclear. However, one explanation is that the inflammatory cells and pro-inflammatory mediators present in the lungs “spill over” into the systemic circulation (48, 52). This state of chronic low-grade systemic inflammation is thought to contribute to the development of comorbidities of COPD (48, 52).

The contribution of systemic oxidative stress in COPD has also been recognized. There is an increased concentration of $H_2O_2$ in the exhaled breath condensate (EBC) of smokers and patients with COPD compared to non-smokers, and those are further increased during exacerbations (38, 39). In addition,
concentrations of lipid peroxidation products (e.g. 8-isoprostane, 4-hydroxy-2-nonenal, and malondialdehyde (MDA)), LTB4, carbon monoxide, and myeloperoxidase (MPO) have consistently been shown to be elevated in exhaled breath or exhaled breath condensate from patients with COPD (50, 53). Systemic exposure to oxidative stress in COPD is also indicated by increased carbonyl adducts, such as 4-hydroxy-2-nonenal in respiratory and skeletal muscle (54-56). Moreover, systemic markers of oxidative stress such as oxidized low-density lipoprotein, advanced oxidation protein products, and MDA are elevated in COPD patients (57, 58).

In order to combat and neutralize the deleterious effects of ROS-mediated damage, the normal lung has various endogenous antioxidant strategies, which employ both enzymatic and non-enzymatic mechanisms. Within the lung lining fluid, several non-enzymatic antioxidant species exist, which include glutathione (GSH), vitamin C, uric acid, vitamin E, and albumin (59). Enzymatic antioxidant mechanisms include SOD, catalase and Gpx. However, studies have shown that COPD patients have a systemic antioxidant imbalance, including reduced vitamin C, GSH and Gpx (53, 60). Moreover, polymorphisms in extracellular SOD have been associated with reduced lung function and susceptibility to COPD (37).

1.6. COPD and cardiovascular disease

There is evidence showing that patients with COPD have an increased risk of cardiovascular disease (CVD) and thus are at greater risk of dying from cardiovascular causes (48, 61, 62). Comorbid CVD can manifest itself in one or more various disorders such as angina, stroke, arrhythmias, hypertrophy of the heart, and myocardial infarction (MI), and its presence greatly reduces the
survivability of COPD patients (63). Studies have reported that up to 40% of deaths in COPD patients are due to cardiovascular disease (64-67) and more people with mild to moderate COPD die of cardiovascular causes than of respiratory failure (61). Specifically, patients with COPD have a significantly higher risk of acute MI, arrhythmia and congestive heart failure (68). Over 5 years of follow-up and compared with patients without COPD, patients with COPD had higher rates of death, MI, stroke and a higher rate of hospitalization due to heart failure, unstable angina, or arterial revascularization (69). Studies have shown that over 50% of patients hospitalized for AECOPD have a high prevalence of coexisting CVD (70). It has also been demonstrated that cardiovascular risk is even more pronounced, and has a greater effect, during the peri-exacerbation period due to further increases in pulmonary and systemic inflammation. One to five days after a severe exacerbation, the risk of MI increases 2-3 times (8) and subclinical ischaemia might be even more common during these events, as well as during exacerbations of only moderate severity (71). A retrospective review examining 24 hour mortality following AECOPD hospitalization found that approximately 60% of deaths that occurred resulted from cardiovascular causes (72). It has also recently been shown that patients with COPD are at increased risk for stroke and this is even higher (approximately 7 fold) in the weeks following an acute severe exacerbation (9).

1.7 Overview of stroke pathophysiology

In 2013, stroke was the second-leading global cause of death behind heart disease, accounting for 11.8% of total deaths worldwide (73). Moreover, stroke is a leading cause of disability. Indeed, it is estimated that up to 30% of stroke
survivors do not recover full independence, and thus require assistance with self-care for the rest of their lives (73). In 2012, the estimated cost for stroke was $33 billion (USA) and is projected to be $1.52 trillion by 2050 for non-Hispanic whites, $313 billion for Hispanics, and $379 billion for blacks (in 2005 USD) (73). Thus, the personal and economic burden of stroke is staggering.

Ischemic stroke is the most common subtype, accounting for approximately 80% of all strokes. This type of stroke typically occurs as a result of a blockage of a cerebral blood vessel by a thrombotic (usually an atherosclerotic plaque) or embolic clot, or as a result of cerebral vascular insufficiency due to structural (e.g. atherosclerosis) and/or functional abnormalities of cerebral blood vessels. Ischemic stroke can be further classified depending on the etiology such as large-artery atherothrombosis, cerebral small vessel disease resulting in lacunar stroke, and cardioembolism. Less frequently, stroke can occur as a result of hemorrhage (intracerebral ~10% or subarachnoid ~3%) or cardiac arrest. There are number of traditional risk factors for stroke. Some stroke risk factors cannot be modified, for example age, genetic predisposition, gender (male), and race, whereas others are potentially modifiable. These include hypertension, hypercholesterolemia, atrial fibrillation, diabetes, and smoking, which account for >60% of stroke risk and often co-exist (74). Moreover, as discussed above, lung diseases including COPD are emerging as ‘novel’ stroke risk factors.

The pathogenesis of ischemic stroke is very complex. In brain tissue of the ischemic core, which is a region characterized by a severe reduction in cerebral blood flow, cell death occurs rapidly and largely as a result of energy failure and subsequent necrotic death (75). Injury to brain tissue surrounding
the infarct core (the ischemic penumbra), however, occurs over hours to days and multiple mechanisms are involved. These include excitotoxicity, calcium dysregulation, mitochondrial dysfunction, spreading depolarizations, and apoptotic cell death (75, 76). Oxidative and nitrosative stress also play a key role in injury development in this region (77). Compelling evidence implicates the ROS-generating NADPH oxidases as key drivers of oxidative stress-induced brain and vascular injury following cerebral ischaemia (78-82). Substantial evidence also supports the importance of inflammation and immune system activation in injury development and expansion after stroke (83, 84). Moreover, there is a growing appreciation of the vascular contribution, particularly at the level of the neurovascular unit (85). The neurovascular unit is a collective term for the structural and functional association between neurons, perivascular astrocytes, vascular smooth muscle cells (pericytes/astrocytes), endothelial cells, and the basal lamina (86). Together, the components of the neurovascular unit act to regulate and maintain cerebral perfusion, preserve homeostatic balance in the brain, and control immune regulation. Furthermore, it represents the primary site of the blood-brain barrier (BBB). Cerebral ischaemia has devastating effects on both the structure and functioning of the neurovascular unit. It impairs endothelial function and thus brain perfusion, disrupts the BBB by increasing its permeability, and enhances inflammatory cell infiltration (85). Collectively, these mechanisms contribute to and exacerbate brain injury (85).

During intracerebral hemorrhage, the most common type of haemorrhage stroke, the accumulation of blood within the brain leads to rapid damage as a result of mechanical injury and increased pressure (87). Secondary damage can also occur due to the presence of intraparenchymal blood. Similar to ischemic
stroke, multiple pathological pathways are involved including excitotoxicity, oxidative stress, inflammation, cytotoxicity of blood, hypermetabolism, and disruption of the neurovascular unit and BBB (88).

1.8 Evidence linking lung function, COPD and stroke

1.8.1 Link between poor lung function and risk of cerebral events

Studies have shown that impairment in lung function is related to an increased risk of stroke (89-93). Previous studies have shown that reduced forced expiratory volume in 1 second (FEV₁) is associated with an increased incidence of both ischemic and haemorrhagic stroke, and this association is independent of smoking status. Similar associations have been observed linking reduced pulmonary function and higher risk of subclinical cerebrovascular abnormalities, including in individuals who have never smoked (94, 95). These asymptomatic lesions, such as silent lacunar infarcts, white matter lesions, and cerebral microbleeds are considered to be precursors of clinical stroke and manifestations of cerebral small vessel disease (96-98). Additionally, associations between lower FEV₁ and markers of subclinical atherosclerosis have been reported, although the relevance of this to the presence of subclinical infarcts and white matter lesions is unclear (99). The explanations for these observations are unclear, although impairments in lung function and lung volume may reflect impairments in cardiac function (100, 101).
1.8.2 COPD and risk of clinical stroke

Recent studies have shown that strokes are more prevalent in COPD compared to the general population (102-104). COPD patients are reported to have an increased risk of approximately 20% for both ischemic and haemorrhagic strokes (9, 68, 105). This risk is estimated to be up to 7-fold higher following an AECOPD compared to stable COPD(9), suggesting that COPD itself is contributing to an increase in stroke risk, as opposed to the risk being solely due to shared risk factors. Consistent with this, studies have shown that chronic inflammatory airway disease (CIAD) is an independent risk factor for long-term mortality post-stroke (106). It is also known that stroke causes lung injury/dysfunction per se as evidenced by impaired cough, weakness of respiratory muscles and increase in the propensity of pneumonia (107-110). Therefore, it is plausible that worsening of lung function due to stroke could contribute to the increase in long-term mortality after stroke.

1.9 Potential mechanisms linking COPD and stroke risk and severity

1.9.1 Contribution of shared risk factors

The factors linking COPD and stroke risk are currently not fully understood and are likely to be interconnected (Figure 1.1). It is well known that two of the most important risk factors for COPD, chronic cigarette smoking and ageing, are also established risk factors for stroke (111, 112). Thus, the association between COPD and stroke may be largely dependent on these shared risk factors (9). Like other traditional stroke risk factors, ageing and chronic
smoking increase the propensity to stroke by impairing the ability of the cerebral circulation to meet the brain’s high-energy demands. This largely occurs as a result of structural and functional changes to cerebral blood vessels, resulting in vascular insufficiency and ultimately brain injury. For example, both risk factors often alter the structure of intra-cranial and extra-cranial blood vessels by promoting atherosclerosis, vascular atrophy and remodeling, and vascular stiffness (113-117). Moreover, these structural abnormalities are typically accompanied by functional impairments of cerebral blood vessels resulting in alterations in cerebral blood flow regulation. Indeed, it is well documented that smoking (and nicotine) and ageing cause endothelial dysfunction (118-124), which in turn, is associated with an increased risk of stroke (125, 126). Also, they impair neurovascular coupling (127-130), which is an essential adaptive mechanism that matches cerebral blood flow to neuronal activity. Lastly, ageing and smoking can disrupt the BBB (131-133), which may contribute to the increased risk of intracerebral haemorrhage and microbleeds in COPD.

Evidence indicates that ageing and smoking produce vascular impairments, at least in part, by promoting oxidative stress, which is driven primarily by the NADPH oxidases (122, 123, 127). Perhaps the best-characterized mechanism by which oxidative stress can cause vascular dysfunction is via the inactivation of endothelial-derived NO by $O_2^-$ (134). This reaction reduces the bioavailability of NO and thus nullifies its vasodilator, anti-platelet, anti-proliferative, and anti-inflammatory properties. In addition, ROS can directly promote inflammation in the vessel wall by inducing the production of cytokines and pro-inflammatory genes through the activation of NF-κβ (135). Importantly, whereas oxidative stress may set the stage for inflammation, it in
turn accentuates ROS production, creating a vicious cycle that worsens vascular dysfunction (76). Indeed, pro-inflammatory cytokines such as TNFα and IL-6 alter the functioning of cerebral vessels by increasing ROS production via the NADPH oxidases (136, 137). Moreover, studies of systemic arteries infer that T-cells and macrophages also contribute (138, 139). Oxidative stress and inflammation can also alter the structure of cerebral vessels by promoting vascular remodeling, stiffness, atherosclerosis, and BBB disruption (76, 140-142).

In addition to producing vascular insufficiency, it is likely that ageing and chronic smoking modulate stroke risk by increasing the propensity for atherosclerotic plaque rupture (143). The pro-thrombotic effects of smoking are well documented. For example, smoking increases platelet activation and triggers the coagulation cascade (144, 145). Similarly, ageing is associated with increased platelet aggregation and enhanced thrombosis (146, 147). Thus, ageing and smoking increase the risk of thrombotic/embolic events.

1.9.2 Association with traditional stroke risk factors

Some but not all studies have shown that an association between COPD and stroke still exists after adjusting for age and smoking status (9, 98, 105). Thus, although it is difficult to correct for the total amount of smoking or environmental smoke exposure (7, 148), stroke risk in COPD might not be wholly explained by the contribution of shared risk factors. As discussed above, multiple studies have shown a link between COPD and the development of cardiovascular disease. Moreover, vascular/stroke risk factors are common in COPD patients including hypertension, diabetes, and hypercholesterolemia (149,
150). Similar to ageing and smoking, these traditional risk factors increase the propensity to stroke by altering the structure (e.g. atherosclerosis and vascular remodelling) and functioning of vessels, and by increasing the propensity for atherosclerotic plaque rupture and thrombus formation (134, 140). Moreover, oxidative (via the NADPH oxidases) and inflammatory mechanisms play vital roles in disease progression (151-159). Thus, although the potential contributions of ageing and smoking cannot be ignored (7), it is conceivable that the systemic inflammation and oxidative stress in COPD may initiate and/or accelerate the development of traditional stroke risk factors, thereby leading to increased stroke risk.

1.9.3. COPD-specific systemic inflammation and oxidative stress

Systemic inflammation is emerging as a non-traditional risk factor for stroke (160, 161). For example, systemic markers of inflammation such as CRP and total leucocyte counts, which are both elevated in COPD, are predictive markers of ischemic stroke risk (162). As discussed, inflammation and oxidative stress are major drivers of cerebral vascular dysfunction. Thus, although definitive proof is lacking, it is conceivable that the systemic inflammation and increased oxidative stress in COPD may independently increase stroke risk by directly promoting cerebral vascular dysfunction and thus vascular insufficiency. Consistent with this concept, COPD is associated with increased carotid-femoral pulse wave velocity (PWV, the “gold standard” measurement of arterial stiffness) independent of cigarette smoke exposure (7, 163, 164). Treatment of COPD patients with an antioxidant cocktail (Vitamin C, Vitamin E, and α-lipoic acid) improves PWV implicating a role for oxidative stress. In COPD patients with
frequent exacerbations, arterial stiffness increases and this is associated with inflammation (71). Importantly, PWV is closely associated with lacunar stroke and white matter lesions (165), which as mentioned are key manifestations of cerebral small vessel disease. Functional abnormalities of systemic arteries have also been reported in COPD patients compared with control subjects and smokers with normal lung function. These include impaired flow-mediated dilation (164, 166), a mechanism that is largely dependent on the production of NO by the endothelium. Evidence suggests that impairments in flow-mediated dilation are related to CRP levels but not pack-years of smoking, and that CRP levels are an independent predictor of flow-mediated dilation, suggesting a role for inflammation (166). Moreover, an antioxidant cocktail improves flow-mediated dilation in COPD patients, implicating a role for oxidative stress (164).

Our knowledge of cerebral artery function in COPD lags behind those studies of systemic arteries. However, evidence thus far suggests that COPD is associated with cerebral vascular disturbances. For example, in an experimental model of COPD, activation of endothelial-dependent dilator pathways paradoxically leads to constriction of cerebral vessels (e.g. middle cerebral artery), indicative of endothelial dysfunction (167). However, the roles of inflammation and oxidative stress in this dysfunction were not examined. Studies measuring cerebral blood flow in COPD patients have revealed contradictory findings. Indeed, some investigators have revealed that cerebral blood flow is reduced in COPD patients (168, 169), whereas others report that it is increased (170, 171). Other studies have focused on examining acute responses to hypercapnia in COPD patients. It is well documented that in healthy subjects, increased partial pressure of carbon dioxide (PaCO₂) results in
cerebral vascular dilation and increased cerebral blood flow. Several mechanisms are responsible including a dilatory response of cerebral arteries, which is largely dependent on NO production. Some but not all studies report that COPD patients show decreased sensitivity to hypercapnia (57, 168, 172), inferring that NO-dependent cerebral vasodilator responses might be impaired. Consistent with this, one study found that these abnormalities were eliminated after adjustments were made for markers of oxidative stress, which might suggest a role for oxidative inactivation of NO (57). However, it is important to remember that central chemoreceptors and the ventilatory response are also involved in hypercapnia cerebral vascular responses. Thus, it is conceivable that impairments of these mechanisms might also contribute. Clearly, more research is needed to fully investigate the impact of COPD (independent of smoking and ageing) on the functioning of cerebral vessels, and how any such abnormalities relate to stroke risk.

Recent evidence suggests that patients with COPD have increased platelet activation, with further activation occurring during AECOPD (173). CRP levels positively correlate with activation of the coagulation/fibrinolysis system after stroke, suggesting a link between coagulation and inflammation (174). Also, excess levels of ROS such as H$_2$O$_2$ may lead to platelet hyperactivity and pro-thrombotic phenotype (146). Thus, COPD-specific inflammation and oxidative stress may also influence stroke risk by increasing susceptibility to thrombotic or embolic events.

The link between acute infections and stroke is well documented. Indeed, numerous studies have shown that acute/chronic viral and bacterial infections are independent risk factors (161, 175). Moreover, this mainly relates to acute
respiratory infections (176). Multiple links between inflammation and coagulation may explain the link between infections and stroke per se (161, 175). Thus, given systemic inflammation is elevated even further during an acute exacerbation; it is likely that such mechanisms may also underpin the increased stroke risk in COPD patients in the weeks following AECOPD.

Figure 1.1: Increased oxidative stress and lung inflammation in response to cigarette smoke causes a spill-over of cytokines (e.g. IL-6, TNFα, SAA) into the systemic circulation. Systemic inflammation in COPD initiates and/or worsens comorbid conditions such as cardiovascular disease (CVD)/traditional stroke risk factors and stroke. Viral and bacterial pathogens markedly increase reactive oxygen species production and systemic inflammation and hence exacerbate COPD and its comorbidities. Targeted co-inhibition of mechanisms underlying both COPD and stroke (e.g. oxidative stress, local and systemic inflammation) may lead to increased survival and improvements in quality of life of patients.
1.10 COPD and stroke outcomes

While many studies have focused on the increased risk of stroke in COPD, few studies have explored whether subjects with COPD have more severe strokes and hence poorer stroke outcomes. There is evidence to support a relationship between systemic inflammation and poor outcome in stroke patients and in models of experimental stroke. Indeed, experimental models of stroke have shown that systemic inflammatory challenges exacerbate brain damage and worsen functional deficits by augmenting cerebral vascular inflammation, BBB disruption, brain oedema, and excitotoxicity (177-179). Moreover, systemic inflammation activates microglia (the brain's resident immune cell) to induce cyclooxygenase-dependent neuroinflammation and increased O$_2^\cdot$ production (180). The mortality rate in stroke patients has been shown to be higher in those with COPD than non-COPD patients, and COPD is an independent predictor of mortality following a stroke (10). Another study has found that exacerbations of COPD impact on stroke outcomes. Patients who were recently diagnosed with an exacerbation of COPD were more at risk of adverse events following a stroke, compared to those without COPD and those with stable COPD (11). No mechanistic link has been established between COPD and worse strokes, however it is plausible that heightened levels of systemic inflammation and oxidative stress may contribute to patients' susceptibility to brain injury and post-stroke adverse events.

1.11 Lung injury after stroke

There is a growing body of evidence to suggest that patients are susceptible to pulmonary complications following a stroke, with one of the most
common complications being pneumonia (181). Pneumonia increases short-term mortality after stroke by approximately 3-fold (182), and is predictably a greater concern for those with impaired lung function, such as patients with COPD. Both clinical and experimental stroke induce an inflammatory response in the brain. Inflammatory products from the brain may spill over, through the damaged BBB, and produce a systemic inflammatory response. Studies have shown that inflammatory changes in the brain are mirrored in the periphery after experimental stroke in mice (183, 184). Following the initial elevation in systemic inflammation, systemic immunosuppression occurs. It is feasible that these peripheral inflammatory changes may affect the immune response of the lungs, thereby putting the patient at risk of pneumonia. Acute lung injury (ALI) is increasingly becoming recognized as a significant contributor to mortality in patients that survive subarachnoid haemorrhage (SAH) (185). Although there is evidence of ALI in SAH, relatively little is known about ALI following ischemic stroke, which make up approximately 80% of all strokes.

1.12 Aims of this thesis

The primary aims of this thesis were to investigate whether COPD impacts on stroke outcomes, and inversely, whether stroke causes acute lung injury in mice. In previous studies conducted in our laboratory, BALB/c mice have been used to model COPD, while C57BL/6 mice have been used for studies of ischaemic stroke. Thus, a strain comparison study was necessary to determine the appropriate mouse strain for studies investigating the link between COPD and stroke. More specifically, this project aimed:
1. To investigate strain-dependent differences between BALB/c and C57BL/6 mice in response to an experimental model of transient ischaemic stroke (Chapter 3).

2. To investigate changes in markers of lung injury in mice following experimental stroke in C57BL/6 mice (Chapter 4).

3. To investigate changes in stroke outcomes in C57BL/6 mice exposed acutely and chronically to cigarette smoke (preclinical mouse model of COPD) (Chapter 5).
Chapter 2

General methods
2.0 Methods

The general methods chapter comprises of methods that have been
applied over multiple chapters within this thesis. Methods specific to individual
chapters are outlined in full in those chapters.

2.1 Animals and ethics

All experimental procedures were conducted in accordance with the
National Health & Medical Research Council (NHMRC) of Australia guidelines for
the care and use of animals for research and were approved by the RMIT
University Animal Ethics Committee (AEC #1532, AEC #1349, AEC #1518). Male
BALB/c and C57BL/6 mice 7-12 weeks of age were obtained from the Animal
Resources Centre (ARC; Perth, Australia) and used for the experiments described
below. On arrival from the ARC, mice were allowed to acclimatize for a minimum
of 2 days before commencement of the experimental protocols described below.
During the acclimatization and experimental periods, mice had access to water
and standard chow ad libitum, and were housed under a 12 h light/ 12 h dark
cycle.

2.2 Comparison of stroke outcome in two mouse strains: BALB/c
and C57BL/6

Male 7- 12 week BALB/c or C57BL/6 mice were randomly assigned to
either sham or stroke surgery groups after 1 week. Mice were weighed prior to
surgery and the day following surgery. Stroke surgeries were performed as
described below with an ischaemic period of either 30 or 50 minutes. Mice were
culled 24 h post-surgery by anaesthetic overdose with isoflurane and the brains
were rapidly removed and used for analysis including infarct and oedema analysis as described below.

2.2.1 Focal cerebral ischaemia and reperfusion via transient middle cerebral artery occlusion (tMCAO)

The transient middle cerebral artery occlusion (tMCAO) model was used to induce stroke in mice. This is the best-characterized model of stroke in rodents (186). Mice were anaesthetized with a mixture of ketamine (150mg/kg, i.p.) and xylazine (10mg/kg, i.p.) in a total volume of 0.2 ml. The level of anaesthesia was monitored throughout surgery, by closely monitoring respiration rate, and toe-pinch response. Once anaesthetized, the head and neck were shaved and cleaned with betadine surgical scrub. An incision was made on the neck and on the skull, and a laser-Doppler probe (Perimed, Sweden) was fixed to the skull to monitor regional cerebral blood flow (rCBF) in the area of the cortex supplied by the middle cerebral artery (~2 mm posterior and 5 mm lateral to bregma). With the use of a dissecting microscope, the common carotid artery was dissected free. A clamp was placed on the common carotid artery and an incision made on the external carotid artery to allow for insertion of the filament. A 6-0 nylon monofilament with a silicone-coated tip (Doccol CO., Redlands, CA, USA) was then inserted into the external carotid artery and then advanced into the internal carotid artery until it reached the origin of the MCA (Figure 2.1). Correct positioning of the filament was confirmed by a >70% reduction in rCBF (Figure 2.2). The filament was left in place to induce ischaemia for a period of 30, 40 or 50 minutes (see individual chapters for specific ischemia duration), and then retracted to allow reperfusion to occur. CBF as measured for
10 minutes during reperfusion. Both the microtip and Laser-Doppler probe were then removed. Prior to the closure of wounds, bupivacaine (0.5% at 6 mg/kg) was applied to wound edges. The wounds were then closed with surgical sutures (6-0 suture silk, B.Braun, Australia). For sham surgery, the carotid artery was visualized but no filament was inserted. Body temperature was monitored throughout surgery using a rectal probe (Testronics, Australia) and maintained at 37°C using a heat lamp (Exo Terra, USA) until mice regained consciousness.

Figure 2.1: Path of filament and position of Laser Doppler probe in tMCAO model.
Figure 2.2: Expected changes in regional cerebral blood flow (rCBF) during tMCAO. A drop in rCBF of > 70% is expected to occur at time of filament insertion, and should remain steady for the duration of ischaemia. Upon withdrawal of the filament, rCBF should return to approximately 80% of pre-ischaemia levels.

2.2.2 Neurological scoring & Functional tests

Prior to mice being culled (24 h or 72 h post-stroke, see individual chapters for specific timepoints), neurological assessment was performed using a 5-point scoring system, and motor impairment was assessed using the hanging wire grip test as previously published (187, 188). Briefly, neurological deficit scoring involved a score from 0 to 4 being assigned to each mouse according to the following criteria: 0 = normal motor function; 1 = flexion of torso and contralateral forelimb when lifted by the tail; 2 = circling to contralateral side when held loosely by the tail on a flat surface with normal posture; 3 = leaning on the contralateral side when at rest; 4 = uncontrolled circling or no
spontaneous movement at rest. To assess motor impairment, a hanging wire test was used to assess forelimb grip strength (79). Briefly, this test involves a horizontal wire secured 30 cm above a padded surface. Mice were suspended by their forelimbs on this wire. The time spent suspended on the wire was recorded and the average hanging time of 3 trials with 5 min rests in-between was calculated. A score of zero was assigned to those mice that fell immediately and a score of 60 was assigned to animals that did not fall.

2.2.3 Quantification of infarct and oedema volumes

Brains were collected from each mouse following euthanasia and frozen over liquid nitrogen 6 h or 24 h after induction of stroke. Coronal sections (30 μm thickness; 420 μm apart) were cut using a cryostat (Leica Microsystems, Germany) and thaw mounted onto glass slides coated with poly-l-lysine (Thermo Scientific, USA). To quantify infarct volumes, tissue-mounted slides were stained with 0.1% thionin. Thionin-stained slides were then imaged and infarct volumes quantified using ImageJ analysis software by correcting for oedema volumes using the following formula: CIV = [RIA – (RHA – LHA)] (thickness of section + distance between sections), where CIV is corrected infarct volume, RIA is right hemisphere infarct area, RHA is right hemisphere area and LHA is left hemisphere area. Oedema volumes (OV) are calculated using OV = (RHA – LHA) thickness of section.

2.3 Effect of stroke on lung inflammation and lung injury

Male 7 – 12 week old C57BL/6 mice were randomly assigned to either sham or stroke surgery groups. Sham or stroke surgeries were performed as described above with an ischaemic period of 50 minutes. Mice were culled at 6
h, 24 h or 72 h post-stroke by an anaesthetic overdose (sodium pentobarbital, 240 mg/kg). Blood was collected from the abdominal vena cava, transferred into a Microvette® collection tube (Sarstedt, Germany), and spun at 10,000 rpm for 5 min. Serum was stored at -80 °C for later analysis. The lungs were then lavaged as described below, perfused free of blood via right ventricular perfusion with 5 ml of PBS, rapidly excised en bloc, rinsed in PBS, blotted, snap frozen in liquid nitrogen and stored at -80 °C until required for PCR analysis. Brains were rapidly removed and frozen over liquid nitrogen, then stored at -80 °C for subsequent infarct and oedema analysis.

2.3.1 Bronchoalveolar lavage and differential cell counts

Lungs were lavaged in situ with a 400 μl aliquot of PBS, followed by three 300 μl aliquots as previously described (189). In total up to 1 ml of bronchoalveolar lavage fluid (BALF) was retrieved per mouse. The total number of viable cells in the BALF was determined using the fluorophores ethidium bromide and acridine orange (AO/EB), on a Nikon Eclipse E600 (Nikon Instruments, USA). Cytospins were prepared using 100 μl of BALF spun at 400 rpm for 10 min using a Cytospin 3 (Shandon, UK). Cytospin preparations were then stained with DiffQuik (Dade Baxter, Australia), and 500 cells per slide were counted and differentiated into macrophages, neutrophils and lymphocytes using standard morphological criteria. The remaining BALF was centrifuged and the supernatant stored at -80 °C until required for further analysis.

2.4 Effect of cigarette smoke exposure on stroke outcome

Cigarette smoke (CS) exposure was used as a validated pre-clinical mouse model of COPD. Male 7-12 week old C57BL/6 mice were randomly assigned to
either sham (room air) or CS exposure groups. Mice were then placed in an 18-L perspex chamber (The Plastic Man, Huntingdale, Victoria, Australia) in a standard chemical hood and exposed to CS generated from 9 cigarettes per day for 2, 8 and 12 weeks as previously described (189). Briefly, mice were exposed to CS generated from 9 cigarettes/day for 2, 8 and 12 weeks (Monday to Friday but not Saturday and Sunday), delivered three times per day at 9 AM, 12 noon and 3 PM with 3 cigarettes spaced over 1 h. Smoke was generated in 50-ml tidal volumes over 10 s, by use of timed draw-back mimicking normal smoking inhalation volume and cigarette burn rate. The mean total suspended particulate mass concentration in the chamber containing CS was ~420 mg m\(^{-3}\) (189). Commercially available filter-tipped Winfield Red cigarettes (manufactured by Philip Morris, Australia) of the following composition were used: 16 mg or less of tar, 1.2 mg or less of nicotine, and 15 mg or less of CO. Sham-exposed mice were placed in an 18-L perspex chamber but were not exposed to CS.

After a CS-exposure period of 2, 8 or 12 weeks, mice were randomly allocated to sham or stroke surgery groups. Sham or stroke surgeries were performed with an ischaemic period of 40 min or 50 min as described above. Mice were culled at 24 h post-stroke by an anaesthetic overdose (sodium pentobarbital, 240 mg/kg). Brains were rapidly removed and frozen over liquid nitrogen, then stored at -80 °C for subsequent analysis. Blood was collected from the abdominal vena cava, transferred into a Microvette® collection tube (Sarstedt, Germany), and spun at 10,000 rpm for 5 min. Serum was stored at -80°C for later analysis. The lungs were then lavaged as described above, perfused free of blood via right ventricular perfusion with 5 ml of PBS, rapidly excised en bloc, rinsed in PBS, blotted, snap frozen in liquid nitrogen and stored at -80 °C.
until required for PCR analysis. Brains were rapidly removed and frozen over liquid nitrogen, then stored at -80 °C for subsequent infarct and oedema analysis.

### 2.4.1 RNA extraction

Lungs from individual mice were crushed to a fine powder in liquid nitrogen with a mortar and pestle, and subsequently homogenised by passing 5 times through a 21G needle with a 1 ml syringe. Total RNA was extracted from lung samples using an RNeasy Plus kit (QIAGEN, Australia), according to the manufacturer's instructions. RNA yield and purity were quantified using a nanodrop (ND-1000, Biolab). Total RNA from lung samples were reverse transcribed to cDNA (Applied Biosystems High Capacity RNA-to-cDNA Kit, USA). Quantitative polymerase chain reaction (qPCR) was performed using mouse-specific TaqMan® Gene Expression Assays (Applied Biosystems, USA), on an ABI 7900HT Sequence Detection System. Samples were assayed in triplicate and negative reverse-transcriptase controls were included. Fold change was determined in comparison to the sham control group, after standardising to GAPDH (housekeeping gene), using the standard Delta-Delta C_{T} method (190).

### 2.5 Data and statistical analysis

All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Version 6.07, La Jolla, CA, USA). Results are presented as mean ± SEM and a $P<0.05$ was considered to be statistically significant. Mouse weights were compared with two-way ANOVA, repeated measures with Bonferroni post-hoc test. Infarct and oedema volumes, BALF, and qPCR were analysed using an unpaired t-test. See experimental chapters for specific statistical analyses of data.
Chapter 3

Comparison of stroke outcome in two mouse strains: BALB/c and C57BL/6
3.1 Introduction

The mechanisms underlying stroke have been studied in a number of animal models (191); however there has been little success in the development of treatments and neuro-protective agents for human disease. Many treatments that have shown effectiveness in animal models have proved to be ineffective in human trials (192). The success of stroke studies depends upon the selection of the most appropriate animal model, for results to be translatable to human disease. There are countless factors that contribute to the selection of the most suitable animal model for any given experiment. There are a number of established animal models used for studying the mechanisms of stroke, each with their own advantages and limitations (191, 193). Ischaemic stroke, the most common subtype of human stroke (194), can be modeled in animals by transiently or permanently blocking blood flow in a cerebral artery. Mice are a popular choice of animal for stroke research for a number of reasons: it is considered more ethical to use smaller rodents over other species; the maintenance cost of mice is comparatively low; reagents for mouse molecular work are cheaper; and their response to ischaemic stroke is well characterized (186).

The choice of strain of mouse is important for studies exploring the mechanisms underlying stroke. Susceptibility to damage from cerebral ischaemia varies between strains of mice. Barone et al (195) showed significant differences in cerebral infarct volumes in response to cerebral ischaemia, between B6D2F1 (BDF1), Swiss Webster (CFW) and BALB/c mouse strains, demonstrating that the BALB/c mouse strain had an increased degree of cerebral
damage following focal ischaemia. The C57BL/6 mouse strain appears to be the most susceptible to cerebral ischaemia, with highly reproducible lesions (196).

To effectively study how COPD impacts upon stroke outcomes, an animal model must be used that effectively mimics the relevant aspects of COPD in humans, while also being a relevant model of experimental stroke. The BALB/c mouse strain is typically used in COPD research, as the response to cigarette smoke (the major risk factor for the development of COPD) mimics many aspects of human disease, such as chronic lung inflammation and impaired lung function (189, 197). Although C57BL/6 mice are widely used in experimental stroke research (198), no study has yet compared cerebral damage after ischaemic stroke between C57BL/6 mice and BALB/c mice.

Therefore, the aim of this experimental chapter was to use the tMCAO model of stroke to compare stroke outcomes (neurological and functional impairment, infarct and oedema volumes) in C57BL/6 and BALB/c mice to determine which strain of mouse should be used for the experiments described in Chapter 5 (i.e. the impact of cigarette smoke exposure on stroke outcomes). It was hypothesised that BALB/c mice would have worse stroke outcomes compared to C57BL/6 mice, and that a shorter period of ischaemia would be required to elicit the ideal infarct volume of 35 – 40 mm³. This volume is ideal as both increases and decreases in infarct volume can be observed (199). BALB/c mice were expected to have worse stroke outcomes based on previous that suggest BALB/c mice are more susceptible to brain injury following ischemic stroke when compared to other strains, largely due to variations in cerebrovascular anatomy.
3.2 Materials and methods

Common materials and solutions are listed in General Methods (Chapter 2).

3.2.1 Animals and ethics

All experimental procedures were approved by the Animal Ethics Committee at RMIT University (AEC #1518). C57BL/6 mice 7-12 weeks of age were obtained from the Animal Resources Centre (ARC; Perth, Australia) and used for the experiments described below.

3.2.2 Experimental protocols

C57BL/6 mice and BALB/c mice underwent tMCAO surgery with an ischaemic period of 30 min (n = 18 for BALB/c; n = 20 for C57BL/6) or 50 min (n = 10 for BALB/c; n = 16 for C57BL/6) as outlined in Chapter 2: General Methods. Regional cerebral blood flow was measured during the procedure for all stroke animals, using trans-cranial laser-Doppler flowmetry. Sham animals (C57BL/6, n = 7) underwent the same procedure except the filament was not inserted. Functional and neurological tests were performed as described in Chapter 2: General Methods, just prior to culling. Mice were culled via anaesthetic overdose with isoflurane, 24 h following the induction of ischaemia. Samples (lungs and brain) were collected as described in Chapter 2: General Methods.

3.3 Results

3.3.1 Degree of hypoperfusion after stroke

Following insertion of the monofilament at the origin of the MCA, all stroke mice experienced a similar drop (~75%) in regional cerebral blood flow
(Figure 3.1). This remained steady over the ischaemic period for all groups. Both strains of mice had a similar degree of reperfusion after 30 min of ischaemia (~80% of initial blood flow in C57BL/6 mice, ~90% in BALB/c mice), and a similar degree of reperfusion after 50 min of ischaemia (~100% of initial blood flow in C57BL/6 mice, ~125% of initial blood flow in BALB/c mice).

Figure 3.1: Regional cerebral blood flow during tMCAO surgery and during reperfusion. Ischaemic periods of 30 min (A, n = 8 - 9) and 50 min (B, n= 5 - 8) were used. All results expressed as mean ± SEM. (P>0.05, Student’s paired t-test).

3.3.2 Stroke outcomes in C57BL/6 and BALB/c mice

Stroke mice with an ischaemic period of 30 min had reduced hanging grip time when compared to sham animals, but this was not significant, and there was no difference between mouse strains (Figure 3.2A). Both the C57BL/6 and BALB/c strains of mice displayed a significant neurological deficit after a stroke with an ischaemic period of 30 min, compared to sham animals (Figure 3.2B, P<0.05). However, BALB/c mice were significantly less neurologically impaired compared to C57BL/6 mice (Figure 3.2B, P<0.05). C57BL/6 mice have a
mortality rate of 8-10% following tMCAO surgery in studies previously conducted in our laboratory. In this study, C57BL/6 mice had a mortality rate of 20% in the 30 min ischaemia group (4/20), and a mortality rate of 19% in the 50 min ischaemic group (3/16). BALB/c mice had a mortality rate of 39% in the 30 min ischaemia group (7/18), and a mortality rate of 40% in the 50 min ischaemia group (4/10).

Figure 3.2: Hanging wire (A) and neurological (B) scores after tMCAO surgery with an ischaemic period of 30 minutes in C57BL/6 mice (n = 8) and BALB/c mice (n = 9), and in C57BL/6 mice after a sham procedure (n = 8). Hanging wire results are expressed as mean ± SEM (one-way ANOVA followed by Bonferroni post-hoc test), neurological deficit score results expressed as median (*P < 0.05 vs. sham, #P 0.05 vs. C57BL/6, Mann-Whitney test)
3.3.3 Cerebral infarct and oedema volumes in C57BL/6 and BALB/c mice

Thionin staining of coronal brain sections 24 h post-stroke showed that BALB/c mice experienced similar cerebral damage compared to C57BL/6 mice (Figure 3.3).

Figure 3.3: Representative thionin-stained coronal brain sections at 24 h post-stroke. BALB/c and C57BL/6 mice underwent tMCAO procedure with an ischaemic period of 30 min or 50 min. Area of infarct is shown circled in yellow.
Cerebral infarct volumes were typically smaller in BALB/c mice (Figure 3.4A & B P>0.05) for both 30 and 50 min ischaemic periods, however this was not significant. The same pattern was also observed for cerebral oedema volumes (Figure 3.4B & D, P>0.05).

Figure 3.4: Cerebral infarct and oedema volumes at 24 h after tMCAO procedure with ischaemic periods of 30 min (A, B; n = 9 for BALB/c, n = 6 for C57BL/6) and 50 min (C, D; n = 5 for BALB/c, n = 9 for C57BL/6). All results are expressed as mean ± SEM (Student’s t-test).

3.4 Discussion

The present study aimed to investigate the strain-dependent differences in response to experimental stroke, in BALB/c and C57BL/6 mice. The purpose of this was to determine the most appropriate strain and duration of ischaemia
to use in subsequent long-term smoke exposure studies. It was expected that BALB/c mice would be more sensitive to cerebral ischaemia than C57BL/6 mice. Previous studies have shown BALB/c mice to have larger infarcts in response to cerebral ischaemia when compared to other mouse strains (195). This may be due to a lack of posterior communicating arteries in the BALB/c mouse brain, which makes at an increased risk of damage but also suggests that they are the most consistent in terms of post-stroke outcomes(195).

This study was limited in that no BALB/c sham mice were included, and so comparisons to sham mice were made to C57BL/6 mice. Thus, there may be strain differences in the hanging wire test that are not shown in this study.

I saw no differences in rCBF between the two strains of mice throughout the stroke surgery. This indicates that both strains of mice had a similar reduction in blood flow, and thus stroke damage should be comparable.

It has been suggested that the sensitivity of the BALB/c mouse strain to cerebral ischaemia is due to a variation in the cerebrovascular anatomy compared to other strains of mice. BALB/c mice have non-existent or non-functional posterior communicating arteries(195), which create a secondary supply of blood to the area of the brain normally supplied by the middle cerebral artery. This lack of collateral blood supply may lead to the development of greater infarcts, and perhaps contributes to the elevated mortality rate in BALB/c mice. It also makes them a more consistent and potentially reliable model of cerebral ischaemia, as the brain is more sensitive to damage.

Despite evidence to suggest BALB/c mice would be more sensitive to cerebral ischaemia, no worsening of stroke outcomes was observed compared to C57BL/6 mice. The only significant difference between the strains of mice was a
lower neurological score in BALB/c mice compared to C57BL/6 mice. BALB/c mice generally had smaller infarct and oedema volumes than C57BL/6 mice, although differences were not significant. This suggests that BALB/c mice may be more resistant to MCAO when compared to C57BL/6 mice. The most significant finding from this study was that BALB/c mice had a high mortality rate after stroke. This may have skewed the results of this study; BALB/c mice that did not survive to 24 h post surgery may have suffered substantial cerebral damage. It may be that the BALB/c mice who survived were resistant to ischaemia, and that BALB/c mice express an ‘all-or-nothing’ response to ischaemia. This present study has shown that BALB/c mice would be an inappropriate choice of mouse strain for a long-term smoke-exposure study, due to the mortality rates observed. C57BL/6 mice will thus be used in following studies.
Chapter 4

Effect of stroke on the lung
4.1 Introduction

Pulmonary complications such as pneumonia, acute lung injury (ALI) and neurogenic pulmonary oedema (NPO) frequently occur in the first few weeks following a stroke (181, 185, 200, 201). These complications are a major contributor to morbidity and mortality (202, 203). ALI, an acute and severe hypoxia, is commonly associated with various forms of brain injury, and is estimated to occur in 5 – 30% of patients with subarachnoid haemorrhage, a subtype of stroke (185, 201, 204). The incidence of ALI after ischaemic stroke is unclear, however one study reported acute respiratory distress syndrome (a more severe form of ALI) in 4% of ischaemic stroke patients (205).

NPO is defined as bilateral pulmonary oedema that occurs following a neurological insult, and it has been recognized in various forms of brain injury (206). The mechanisms involved in NPO continue to be debated; however one possible mechanism is the “blast injury theory”. Damage to the central nervous system initiates a massive sympathetic discharge, known as a sympathetic “storm” (207). This leads to systemic vasoconstriction, and subsequently a shift of blood to the pulmonary circulation due to their low resistance. This leads to increased pulmonary capillary pressure, damaging the capillary epithelium and causing pulmonary oedema (204). It is also believed that the over-activation of the sympathetic nervous system initiates a systemic inflammatory response, with infiltration of activated neutrophils and macrophages into the alveolar spaces (204, 208). A “double-hit” model has been proposed to describe the mechanism through which lung failure and pulmonary complications occur in ischaemic stroke (204). The initial sympathetic storm damages the lungs, making them more susceptible to further damage (first hit). Immunosuppression occurs
as early as 12 hours following a stroke, making the lungs more susceptible to infection and pneumonia (second hit).

Currently, studies exploring the time-course of pulmonary inflammation following ischaemic stroke are limited. Inflammatory cell counts in BAL have been measured 24 hours after experimental stroke in mice (209), however 24 hours post-stroke may be too late to observe inflammation leading to lung injury. Levels of inflammatory cytokines such as TNF-α, IL-6 and IL-1β appeared to increase in the lungs of uninfected mice 4 h after stroke (210). It has also been shown that the peripheral inflammatory response induced by stroke, peaks 4 hours after the stroke (184). These findings suggest that an investigation of lung inflammation and injury induced by stroke would need to draw a focus on the hours shortly after a stroke.

This current study aims to investigate inflammatory changes in the lungs following experimental ischaemic stroke in mice, in the period shortly after stroke (6 h) and 24 h following stroke. It was hypothesized that we would see an initial increase in lung inflammation at 6 h, with lung inflammation decreasing by 24 h post-stroke.

4.2 Materials and methods

Common materials and solutions are listed in General Methods (Chapter 2).

4.2.1 Animals and ethics

All experimental procedures were approved by the Animal Ethics Committee at RMIT University (AEC #1349). C57BL/6 mice 7-12 weeks of age
were obtained from the Animal Resources Centre (ARC; Perth, Australia) and used for the experiments described below.

### 4.2.2 Experimental protocols

Mice underwent tMCAO surgery with an ischaemic period of 50 min as outlined in Chapter 2: General Methods. Regional cerebral blood flow was measured during the procedure for all stroke animals, using trans-cranial laser-Doppler flowmetry. Sham animals underwent the same procedure except the filament was not inserted. Functional and neurological tests were performed as described in Chapter 2: General Methods, just prior to culling. Mice were culled via anaesthetic overdose with sodium pentobarbital (240mg/kg), 6 h or 24 h following the induction of ischaemia. Samples (BALf, lungs and brain) were collected as described in Chapter 2: General Methods.

### 4.3 Results

#### 4.3.1 Degree of hypoperfusion after stroke

Following insertion of the monofilament at the origin of the MCA, all stroke mice experienced a similar drop (~75%) in regional cerebral blood flow (Figure 4.1). This remained steady over the ischaemic period for both the 6 h and 24 h groups. A similar degree of reperfusion (~100%) was observed in all mice across the 15 min reperfusion period.
Figure 4.1: Regional cerebral blood flow during tMCAO surgery and during reperfusion. Procedures were performed with an ischaemic period of 50 minutes. All results expressed as mean ± SEM (n = 6 – 9). (P>0.05, Student’s paired t-test).

4.3.2 Functional and neurological outcomes of stroke

Stroke mice displayed significant neurological impairment compared to sham mice 6 h and 24 h post-stroke (Figure 4.2A, P<0.05, two-way ANOVA followed by Bonferroni post-hoc test). Mice did not show a difference in neurological impairment 24 h post-stroke compared to 6 h post-stroke (P>0.05). Stroke mice had a reduction in hanging time on the hanging wire test 24 h post-stroke, compared to sham control mice (Figure 4.2B, P<0.05, Student’s unpaired t-test). Infarct and oedema volumes were measured at 24 h post-stroke (Figure 4.3). Hanging wire, infarct and oedema analyses were not performed at 6 h, as
this time-point is too early to see changes in functional impairment, and the infarct is not close to being completely formed at 6 h, and thus infarct measurements would not be indicative of cerebral damage.
Figure 4.2: Neurological deficit scores (A, n = 6-9) 6 h and 24 h post-stroke or sham surgery, and hanging wire tests (B, n = 8-9) 24 h post-stroke surgery. Hanging wire results are expressed as mean ± SEM (*P < 0.05 vs. sham, Student’s unpaired t-test), neurological deficit score results expressed as median (*P<0.05 vs. sham, Mann-Whitney test).

Figure 4.3: Infarct and oedema volumes at 24 h post-stroke. All results are expressed as mean ± SEM (n = 5).
4.3.3 Effect of stroke on lung inflammation

There was a significant increase in total inflammatory cells and macrophages in the BALF at 6 h following stroke (Figure 4.4, P<0.05, two-way ANOVA followed by Bonferroni post-hoc test). However, there was no increase in the number of neutrophils or lymphocytes in the BALF of mice at 6 h following stroke (Figure 4.4, P>0.05). Interestingly, there was no increase in total cells, macrophages, neutrophils or lymphocytes in BALF of mice 24 h post-stroke compared to sham stroke mice (Figure 4.4, P>0.05). Gene expression of IL-6 and MIP-2α was significantly increased in whole lung tissue of mice 6 h following stroke (Figure 4.5, P<0.05, Student’s unpaired t-test). However, no changes in the expression of IL-1β, TNF-α, MCP-1 and MMP-12 were observed in whole lung tissue 6 h post-stroke (Figure 4.5, P>0.05). Interestingly, there was no difference in IL-6, MIP-2α, IL-1β, TNF-α and MCP-1 in whole lung tissue 24 h post-stroke (Figure 4.5, P>0.05).
Figure 4.4: Inflammatory cells in bronchoalveolar lavage fluid (BALF) at 6 h and 24 h post-stroke. Total (A), macrophage (B), neutrophil (C) and lymphocyte (D) cell counts are shown as mean ± SEM (n = 6 – 9, *P<0.05 vs sham-exposed mice, two-way ANOVA followed by Bonferroni post-hoc test)
Figure 4.5: mRNA expression of proinflammatory genes in whole lung tissue as detected by qPCR, 6 h post-stroke. All results are expressed as mean ± SEM (n = 3-5, *P<0.05 vs sham-exposed mice, Student's unpaired t-test)
Figure 4.6: mRNA expression of proinflammatory genes in whole lung tissue as detected by qPCR, 24 h post-stroke. All results are expressed as mean ± SEM (n = 8, Student's unpaired t-test)

4.4 Discussion

It is well established that ALI and NPO frequently occur in humans after stroke, but the mechanisms underlying this phenomenon are not clearly understood. Therefore, this experimental chapter investigated whether
Experimental stroke in mice can cause lung inflammation. Moreover, this is the first study to characterise any pulmonary changes in this animal model of stroke. In the present study we found that there was an increase in BALF inflammation 6 hours after stroke. This increase in BALF inflammation was overwhelmingly due to an increase in macrophages. Interestingly, we did not see an increase in MCP-1 expression, a key chemoattractant of macrophages, at any time-point in whole lung tissue. This suggests that other chemoattractants were likely to be responsible for the recruitment of macrophages into the lungs.

What was interesting in the current study was that there was a significant increase in the mRNA expression of MIP-2α, a chemokine secreted by inflammatory cells such as macrophages and a homologue of human IL-8 (211), in lung tissue at 6 h post-stroke. It is well known that MIP-2α is involved in initiating neutrophilic inflammation.(212) Neutrophilic inflammation appears to play a key role in the development of ALI (213). Although an increase in neutrophils was not observed in the BALF in this study, there may be evidence of neutrophilic inflammation in lung tissue, and lung samples have been kept for histological analysis. An animal study of traumatic brain injury (TBI) found evidence of neutrophil infiltration and ALI in mouse lung tissue, 12 h and 24 h after brain injury (214). In humans, a study investigating the occurrence of lung injury in patients with fatal brain injury found that expression of IL-8 (a neutrophil chemoattractant) was increased in lung tissue, and an increase in neutrophils was found in the BALF (215). It is interesting to note that while we observed an increase in the neutrophil chemotactic factor MIP-2α, we did not see an increase in neutrophils in the BALF of mice 6 h post-stroke.
In the present study we found that IL-6 mRNA expression in whole lung tissue was increased at 6 h post-stroke, and this was ameliorated by 24 h post-stroke. This finding is in accord with studies showing that the IL-6 is a pro-inflammatory cytokine involved in the development of ALI (216). Increased expression of IL-6 in BALF is a predictor of mortality in ALI (217). Thus, regardless of the mechanisms responsible for the increased BALF inflammation observed 6 hours post-stroke, results from this experimental chapter are similar to what was observed clinically, suggesting that ALI could be occurring in the hours following experimental stroke in mice. It is of importance that this earlier time-point is a focus of further research as this may be when lung damage is occurring after ischaemic stroke. Future studies should include a later time-point (e.g. 72 h) to investigate further changes to the lung inflammatory profile after ischaemic stroke, and to see if inflammatory changes are sustained in the days following ischaemic neurological injury. Immunosuppression occurs after the initial rise in inflammation after stroke, and including a later time-point in future studies will help shed light on how this immunosuppression may contribute to lung injury and susceptibility to infection.

ALI makes the lungs more susceptible to further injury and infection. This can be explained by the “double-hit” theory, which suggests that the lungs are initially “primed”, with an activation and infiltration of inflammatory cells in the lungs. This creates an exaggerated response to a secondary insult such as an infection, leading to further injury (204, 218). Although this hasn’t been investigated in the current study, further characterization of ALI in experimental stroke should involve testing the susceptibility of lungs to infection and injury in the days after stroke.
A common feature of lung injury following neurological damage is NPO. The current theory describing mechanisms leading to NPO is that initially hydrostatic pulmonary oedema occurs (leading to low protein content in pulmonary fluid), and that damage to the pulmonary capillaries persists after normal haemodynamics are restored (leading to high protein content in pulmonary fluid). While we did not measure the protein content of BALF of stroke mice in the current study, it would be useful to measure protein content in the BALF in future studies to investigate the occurrence of NPO after experimental stroke. Determination of lung wet weight to dry weight ratio should also be made in future as a gravimetric assessment of pulmonary oedema (219).

This study is the first to investigate pulmonary inflammatory changes after ischaemic stroke in mice, as an initial investigation into ALI caused by ischaemic stroke. ALI and NPO are of particular importance to those with already compromised lungs, such as COPD patients. Post-stroke mortality appears to be higher in the COPD population compared to the general population (10). Pulmonary complications such as ALI and NPO frequently occur after stroke, and these are likely factors contributing to higher mortality in the COPD population.
Chapter 5

Effect of cigarette smoke exposure on stroke outcome
5.1 Introduction

COPD is increasingly being recognized as a risk factor for stroke. Recent studies show that the risk of stroke is 20% greater in COPD compared to the general population, and this risk is even greater following an AECOPD (220). There is also some evidence to suggest that there is a relationship between COPD and worse stroke outcomes (10, 11). Patients with COPD are at greater risk of adverse events and mortality following a stroke compared to those without COPD. However, no causal mechanism has been established between COPD and worse outcomes following a stroke. As discussed in Chapter 1: Introduction, it is plausible that heightened levels of systemic inflammation and oxidative stress may contribute to increased stroke severity and the occurrence of post-stroke adverse events.

It is difficult to determine the causal mechanisms that may explain worse outcomes following a stroke in human COPD. However, one way to potentially understand the mechanistic links between COPD and stroke outcomes is to use a preclinical model that combines COPD and stroke. It is surprising and interesting that no animal studies have been undertaken investigating stroke in the context of COPD. The preclinical mouse model of COPD used in this study replicates many aspects of human disease, and has been used previously to investigate the systemic changes of COPD (197). Briefly, the model involves exposing mice to cigarette smoke over a period of weeks to months, depending on what features of COPD want to be modeled. Mice begin to develop an inflammatory response to cigarette smoke after 4 days of cigarette smoke exposure, and begin to develop emphysema and a lung phenotype comparable to COPD after 3-6 months of exposure to cigarette smoke (18). This model typically uses BALB/c mice, as they
have a robust response to the cigarette smoke. The current study will investigate stroke outcomes after 2 weeks (acute), 8 weeks (sub-chronic) and 12 weeks (chronic) of cigarette smoke exposure, time-points which represent the initiation (acute), progression (8 weeks) and development (12 weeks) of COPD.

Therefore, the aim of this experimental chapter was to investigate stroke outcomes (functional hanging wire test, neurological scoring, infarct and oedema volume) in an animal model of COPD. It was hypothesized that COPD/smoking will worsen brain injury after ischaemic stroke in association with exacerbated oxidative stress and inflammation.

5.2 Materials and methods

Common materials and solutions are listed in General Methods (Chapter 2).

5.2.1 Animals and ethics

All experimental procedures were approved by the Animal Ethics Committee at RMIT University (AEC #1532). C57BL/6 mice 7-12 weeks of age were obtained from the Animal Resources Centre (ARC; Perth, Australia) and used for the experiments described below.

5.2.1 Experimental Protocols

C57BL/6 mice underwent cigarette smoke (CS) exposure as outlined in Chapter 2: General Methods, for a period of 2 weeks (acute exposure, n = 10 per group), 8 weeks (sub-chronic exposure, n = 18 per group) or 12 weeks (chronic exposure, n = 35 – 47 per group). Following the acute CS exposure period, mice underwent tMCAO surgery with an ischaemic period of 30 min, as outlined in
Chapter 2: General Methods. Regional cerebral blood flow was measured during the procedure for all animals using trans-cranial laser-Doppler flowmetry. Following the sub-chronic and chronic CS exposure periods, mice were randomly assigned to stroke or sham groups. Stroke mice underwent tMCAO surgery with an ischaemic period of 40 min. Sham animals underwent the same procedure except that the filament was not inserted. Functional and neurological tests were performed as described in Chapter 2: General Methods, just prior to culling. Mice were culled via anaesthetic overdose with sodium pentobarbital (240mg/kg), 24 h following the induction of ischaemia. Samples (blood, BALF, lungs and brain) were collected as described in Chapter 2: General Methods. Mice were excluded from analyses if rCBF data showed they did not meet criteria for a ‘successful’ stroke (n = 6 acute exposure; n = 6 sub-chronic exposure; n = 10 chronic exposure), or if they did not survive to 24 h after stroke (n = 1 acute exposure; n = 4 sub-chronic exposure; n = 5 chronic exposure).

5.3 Results

5.3.1 Effect of cigarette smoke exposure on body weight and lung inflammation

Body weight was lower in mice exposed to CS for 2, 8 and 12 weeks compared to sham mice (Figure 5.1, 5.2, 5.3, P<0.05).

Following 2 weeks of CS exposure, significant increases in neutrophils and lymphocytes were observed in BALF (P<0.05, Student’s unpaired t-test), although there was no significant increase in total cell counts or macrophage counts (P>0.05; Figure 5.4). However, following 8 weeks of CS exposure, significant increases in all inflammatory cell types (totals, macrophages,
neutrophils, lymphocytes) were observed in the BALF (P<0.05, two-way ANOVA followed by Bonferroni post-hoc test; Figure 5.5). Stroke did not cause a further increase on inflammatory cell counts, which is consistent with findings in Chapter 4 at 24 h. After 12 weeks of CS exposure, increases in all inflammatory cell types were observed in the BALF, however this was only significant for total cells, macrophages and neutrophils in the smoke + sham surgery group, and only total cell counts in the smoke + stroke surgery group (P<0.05, two-way ANOVA followed by Bonferroni post-hoc test; Figure 5.6).

Figure 5.1: Effect of cigarette smoke exposure on body weight. Mice were exposed to cigarette smoke (n = 10) or sham smoke (n = 10) for 2 weeks. Results are expressed as mean ± SEM (Student’s unpaired t-test performed on final weights, *P<0.05 vs sham-exposed control group).
Figure 5.2: Body weight across 8-week smoke exposure period. Mice were exposed to cigarette smoke (n = 18) or sham smoke (n = 18) for 8 weeks. Results expressed as mean ± SEM (Student’s unpaired t-test performed on final weights, *P<0.05 vs sham-exposed control group).

Figure 5.3: Effect of 12 week smoke exposure on body weight. Mice were exposed to cigarette smoke (n = 47) or sham smoke (n = 35) for 12 weeks. Results expressed as mean ± SEM (Student’s unpaired t-test performed on final weights, *P<0.05 vs sham-exposed control group).
Figure 5.4: Effect of 2 weeks cigarette smoke exposure on inflammatory cells in bronchoalveolar lavage fluid (BALF). Total (A), macrophage (B), neutrophil (C) and lymphocyte (D) cell counts are shown as mean ± SEM (n = 5 – 6, *P<0.05 vs sham-exposed mice, Student’s unpaired t - test).
Figure 5.5: Effect of 8 weeks of cigarette smoke exposure on inflammatory cell counts in bronchoalveolar lavage fluid (BALF). Total (A), macrophage (B), neutrophil (C) and lymphocyte (D) cell counts are shown as mean ± SEM (n = 5-7, *P<0.05 vs sham-exposed control group, two-way ANOVA followed by Bonferroni post-hoc test)
Figure 5.6: Effect of 12 weeks cigarette smoke exposure on inflammatory cell counts in bronchoalveolar lavage fluid (BALF). Total (A), macrophage (B), neutrophil (C) and lymphocyte (D) cell counts are shown as mean ± SEM (n = 4-8, *P<0.05 vs sham-exposed control group, two-way ANOVA followed by Bonferroni post-hoc test)

5.3.2 Degree of hypoperfusion following stroke

Following insertion of the monofilament at the origin of the MCA, all stroke mice experienced a similar drop (~75%) in regional cerebral blood flow (Figures 5.7 – 5.9). This remained steady over the ischaemic period for all groups. A similar degree of reperfusion was observed in all mice in acute (2 weeks), sub-chronic (8 weeks) and chronic (12 week) experiments, though a higher degree of reperfusion was observed in the 12 weeks CS-exposed mice (Figure 5.9, Student's unpaired t-test, P<0.05).
Figure 5.7: Regional cerebral blood flow during tMCAO surgery and during reperfusion, following 2 weeks of cigarette smoke exposure or sham smoke (room air) exposure (n = 8 – 10). (P>0.05, Student’s paired t-test).

Figure 5.8: Regional cerebral blood flow during tMCAO surgery and during reperfusion, following 8 weeks of cigarette smoke exposure or sham smoke (room air) exposure (n = 5 – 7). (P>0.05, Student's paired t-test).
5.3.3 Effect of cigarette smoke exposure on stroke outcomes

All stroke groups displayed some degree of neurological deficit 24 h after stroke. Neurological deficit scores in experimental stroke groups were significantly different from sham surgery control groups in the sub-chronic (8 weeks) and chronic (12 weeks) CS exposure studies (Figure 5.10; P<0.05, Student’s t-test or two-way ANOVA followed by Bonferroni post-hoc test). Cigarette smoke appeared to have no effect on neurological deficit scores after acute, sub-chronic or chronic exposure. All stroke groups had a reduction in hanging time on the hanging wire test, and this was significantly different from sham surgery control groups in the sub-chronic and chronic smoke exposure.
studies (Figure 5.10; P<0.05, Student’s t-test or two-way ANOVA followed by Bonferroni post-hoc test).

Figure 5.10: Neurological deficit (A, B, C) and functional hanging wire (D, E, F) scores 24 h post-stroke or sham surgery. Mice were exposed to 2 (A, D; n = 8-11), 8 (B, E; n= 5 - 8) or 12 (C, F; n = 8 - 18) weeks of cigarette smoke or sham smoke (room air) prior to sham or stroke surgery. All results are expressed as mean ±
SEM. (*P<0.05 vs. sham, Student's t-test or two-way ANOVA followed by Bonferroni post-hoc test).

5.3.4 Effect of cigarette smoke exposure on infarct and oedema

No differences in infarct volume were observed between smoke and sham groups after 2, 8 or 12 weeks of CS exposure. There was a trend for oedema volumes to be lower in smoke exposed groups compared to controls, however this difference was not significant (P>0.05, Figure 5.11).
Figure 5.11: Cerebral infarct and oedema volumes at 24 h after tMCAO procedure. Mice were exposed to 2 (A, D; n = 5 - 10), 8 (B, E; n = 5) or 12 (C, F; n = 6 - 8) weeks of cigarette smoke or sham smoke (room air) prior to stroke surgery. All results are expressed as mean ± SEM (Student’s unpaired t-test).

5.4 Discussion

This experimental chapter investigated stroke outcomes in a mouse model of COPD. It was hypothesized that chronic CS exposure would lead to
worse stroke outcomes in mice. However, this study found that although CS exposure caused its expected effects on body weight (i.e. reduced body weight) and lung inflammation (i.e. increase in lung inflammation), there was no effect on stroke outcomes.

Cigarette smoke exposure causes an immediate inflammatory response in the lungs, by triggering the activation of proinflammatory mediators such as TNF-α NFκB and MMP-12. This results in the recruitment of inflammatory cells such as neutrophils, macrophages and lymphocytes into the lungs, and this immune response contribute to the damage of lung tissue (189). After 2, 8 and 12 weeks of CS exposure inflammatory cells were elevated in the BALF, indicating that that lung inflammation was present in these mice. However, these cell counts after 2 weeks of CS exposure were lower than what has historically been seen in the BALB/c mouse strain in response to CS (189) after 4 days. We have previously shown that the C57BL/6 mouse strain is more resistant to acute CS-induced lung inflammation compared to the BALB/c mouse strain (189, 221). In light of this, we extended the cigarette smoke exposure period out to 8 (sub-chronic protocol) and 12 (chronic protocol) weeks.

Cigarette smoke exposure led to a reduction in body weight across all three time-points (2, 8 and 12 weeks). Nicotine, a primary constituent of CS, is known to suppress appetite. Although we did not measure food intake in our study, we have previously shown that CS-exposed mice eat less (222) However, studies have shown that the weight loss in response to CS is not solely due to a reduction in appetite, but that other metabolic pathways are also involved, such as decreases in energy intake, increases in energy expenditure and accelerated proteolysis (223, 224).
I then went on to explore stroke outcomes in mice that had been exposed to cigarette smoke for 2, 8 and 12 weeks. Despite increased lung inflammation in these CS-exposed mice, it was surprising that we did not see changes in any of the measured stroke outcomes, including neurological deficit score, hanging wire test, infarct and oedema volumes, given that there is increasing evidence to suggest that COPD is associated with worse outcomes following stroke in humans(10). It has been suggested that this may be due to increased systemic inflammation and oxidative stress making the brain more susceptible to brain injury, and the whole body more susceptible to adverse events following a stroke. My data suggests that the mouse model of COPD used in this study (i.e. 2, 8 and 12 weeks of CS exposure) has no effect on stroke severity. There are a number of possible reasons why stroke severity was not worse in this study: (i) 12 weeks of CS exposure may have been insufficient to induce systemic changes believed to play a key role in worsened stroke outcomes, (ii) COPD may not directly lead to worsened stroke severity, but may make an individual more susceptible to post-stroke complications and adverse events, and (iii) COPD may increase risk but play no role in stroke severity.

Another important point to consider is that all mice in this study were culled 24 h post-stroke. At this time-point, the cerebral infarct is almost fully formed (~70-80%) and we can assess stroke severity (225). However, it could be that stroke outcomes were not different in CS-exposed mice because the time at which we assessed stroke outcomes were too soon (i.e 24 hours) and that outcomes should be assessed at later time points (e.g. >72 hours) when the infarct is fully developed(225). Therefore, to assess long-term outcomes after stroke, which are more clinically relevant, future experiments should compare
stroke outcomes in the days and weeks following experimental stroke in COPD mice and non-COPD mice. This will allow us to investigate the mechanisms leading to an increased mortality following a stroke in patients with COPD.

Future experiments will require an assessment of the systemic inflammation and oxidative stress in C57BL/6 mice after CS exposure. Blood samples were collected from mice used in this study; however time was not permitting for an analysis to occur. The CS exposure period may need to be lengthened for C57BL/6 mice, as they are not as sensitive to CS-induced inflammation as the BALB/c mouse strain, and systemic changes may not be occurring after 12 weeks of cigarette smoke exposure.

Finally, it is possible that COPD may not impact on stroke severity, but may just increase the risk of stroke. The model of stroke used in this study cannot be used to investigate risk; however it can be used to investigate mechanisms and physiological changes that would likely increase the risk of a stroke. It is known that CS increases the risk of stroke through a number of mechanisms, such as hypercoagulability and increased immunoreactivity in atherosclerotic plaques. These changes may be sustained in COPD. Future studies will investigate cardiovascular changes in this preclinical animal model of COPD, which may elicit an increased risk of stroke.
Chapter 6

Conclusion
This thesis has systematically examined (i) strain-dependant differences of BALB/c and C57BL/6 mice in response to an experimental model of transient ischaemic stroke (tMCAO) (Chapter 3), (ii) changes in markers of lung injury in mice following experimental stroke (Chapter 4) and (iii) changes in stroke outcomes in C57BL/6 mice exposed acutely and chronically to cigarette smoke (preclinical mouse model of COPD) (Chapter 5).

The purpose of investigating the strain-dependent differences of BALB/c and C57BL/6 mice in response to an experimental model of transient ischaemic stroke (Chapter 3) was to identify the most suitable strain of mouse for the effects of COPD on stroke outcomes (Chapter 5). The BALB/c mouse strain has been used for many COPD studies, as its response to cigarette smoke mimics many aspects of human disease. However, BALB/c mice are not typically used for experimental stroke studies (unlike the use of C57BL/6 mice), and as such we needed to characterize their response to ischaemic stroke prior to a long-term study on the effect of COPD on ischaemic stroke outcomes. Results from the mouse strain comparison study (C57BL/6 vs. BALB/c) demonstrated that there were few differences between the strains in terms of measured stroke outcomes, however the mortality rate in the BALB/c mouse strain was much higher than the C57BL/6 mouse strain. Due to these results, it was decided that the BALB/c mouse strain would be unsuitable for further experiments, and the C56BL/6 mouse strain would be used to study the effect of COPD on stroke outcomes.

Respiratory complications after stroke frequently occur. Acute lung injury (ALI) and neurogenic pulmonary oedema (NPO) have scarcely been studied in the context of ischaemic stroke despite pulmonary complications being a significant contributor to morbidity and mortality. This project investigated
inflammatory changes in the lungs after experimental stroke in mice as a preliminary investigation into the pulmonary changes that occur following ischaemic stroke (Chapter 4). This study found that an increase in lung inflammation occurs shortly after ischaemic stroke in mice. A increase in MIP-2α mRNA expression was observed in lung tissue at 6 h post-stroke, and an increase in macrophages was observed 6 h after stroke. Further work needs to be done to characterize the extent of lung injury that occurs after experimental ischaemic stroke. Lung tissue sections have been kept for histological analysis, to investigate the degree of neutrophil infiltration into the lungs.

This study was the first to investigate outcomes of ischaemic stroke in an animal model of COPD (Chapter 5). An increasing number of studies are showing an association between COPD and risk of stroke, and worse stroke outcomes (9-11, 220). Although the mechanisms underlying increased risk of stroke, and worse stroke outcomes, in COPD are unknown, the effect of COPD on stroke outcomes may in part be due to an increased level of systemic inflammation and oxidative stress in individuals with COPD. It was therefore hypothesized that worse outcomes will be observed after ischaemic stroke in an animal model of COPD, compared to healthy mice. However, the results from this study suggest that this is not the case, as chronic cigarette smoke exposure did not worsen stroke outcomes. A few possible reasons for this could be: (i) the animal model of COPD used in this study may have been insufficient to induce systemic changes believed to play a key role in worsened stroke outcomes, (ii) COPD may not directly lead to worsened stroke severity, but may make an individual more susceptible to post-stroke complications and adverse events, and (iii) COPD may increase risk but play no role in stroke severity. Therefore, future studies should
look at extending the duration of cigarette smoke exposure (i.e. > 12 weeks) for C57BL/6 mice, as these mice are typically not as sensitive to cigarette smoke exposure as the BALB/c mouse strain.

In addition, the bloods collected from this study should be assayed for systemic inflammatory markers and oxidative stress to see if 12 weeks of cigarette smoke was indeed elevating systemic inflammation and oxidative stress in C57BL/6 mice. It could be that we didn’t see worse stroke outcomes after 12 weeks cigarette smoke exposure because this period of smoke exposure was insufficient to elevate systemic inflammation and oxidative stress. In addition, all mice in this study were culled 24 h post-stroke. At this time-point, the cerebral infarct is almost fully formed and we can assess stroke severity. To assess long-term outcomes after stroke, which are more clinically relevant, future experiments should compare stroke outcomes in the days and weeks following experimental stroke in COPD mice and non-COPD mice. This will allow us to investigate the mechanisms leading to an increased mortality following a stroke in patients with COPD.

In conclusion, this project found that (i) BALB/c mice had a high mortality rate in response to tMCAO compared to C57BL/6 mice, (ii) experimental ischaemic stroke leads to an inflammatory response in the lungs, although further work is needed to characterize the pulmonary outcomes of ischaemic stroke, and (iii) the selected preclinical animal model of COPD had no effect on stroke outcomes.
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