EFFECTS OF ANGIOTENSIN RECEPTOR BLOCKERS AND ANGIOTENSIN CONVERTING ENZYME INHIBITORS ON THE HAMSTER TRACHEAL MUCOSAL BARRIERS TO *PSEUDOMONAS AERUGINOSA* INFECTION

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A Thesis Submitted to the University of Zambia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Infectious Diseases

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DEDICATION

This work is dedicated to my family, my children Eliana, Amelia and Zacheriah, my wife Bonnie, and my parents Herbert Mubbunu and Yolanta Mubbunu for their unfailing love and support during my studies.

DECLARATION

I, Lumamba Mubbunu, hereby declare to the Senate of the University of Zambia that this work is my own and that the work of other persons utilized in this thesis has been duly acknowledged. This work presented here has not previously been presented at this university or any other university for similar purposes.

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Signature Date

APPROVAL

This thesis of Lumamba Mubbunu has been approved as fulfilling the requirements for the award of a Doctoral degree in Infectious Diseases by the University of Zambia.

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ABSTRACT

The severe cases of COVID-19 observed in patients with hypertension and diabetes have created controversies as to why this was the case and the role of ARBs, and ACE inhibitors was not ruled out as a contributing factor since the induction of cough is one of their major side effects. Studies have found that the expression level of ACE2 in cells was significantly reduced after COVID-19 infection, leading to an increased level of angiotensin II and activation of the renin-angiotensin-aldosterone system, thus over-activating the angiotensin II type 1 receptor of lung cells to induce and aggravate lung injury. ARBs and ACEIs interfere with the renin-angiotensin aldosterone system in producing their therapeutic effect. The function of gas exchange for the respiratory system exposes it to foreign particles, including infectious agents, allergens, and other substances that can damage it. The role of the airway mucosa is to act as a physical barrier between the external environment and the internal environment. The mucosal barrier function is composed of the mucociliary escalator, intercellular apical junction complexes that regulate paracellular permeability, and antimicrobial peptides secreted by airway epithelial cells. The three components work together to protect the respiratory tract, and impairment of one or more of these components may increase the susceptibility of the respiratory system to infections. Respiratory mucins are very important for the viscoelasticity properties of respiratory mucus, and an increase in the secretion of respiratory mucins can negatively affect the mucociliary escalator. This study aimed to investigate the effects of ACE inhibitors and ARBs on inflammatory mediators and how inflammatory mediators affect tracheal mucosal barriers. The study used a hamster (*Mesocricetus auratus*) as an experimental animal model and ELISA techniques to assess the increase or decrease in Bradykinin, MUC5AC, MUC5B, and FOXA2. Additionally, histological techniques were used to assess goblet cell hyperplasia and hyperplasia of the submucosal glands of the tracheal mucosa. Furthermore, the study challenged hamsters treated with enalapril or losartan with 0.2 ml of pH 7.0 containing 1.5 x 10^8 cfu / ml of *Pseudomonas aeruginosa* to assess the effects of ARB and ACEI on tracheal mucosa barriers. Dunnett's t-test was used to analyze the results and a p-value less than 0.05 was considered significant. Hamsters treated with enalapril, or losartan had an increase in bradykinin, MUC5AC and MUC5B concentration compared to the control, however, the increase was not significant ($p > 0.05$). On the contrary, hamsters treated with enalapril, or Losartan had a decrease in FOXA2

concentration, however, the decrease was also not significant ($p > 0.05$). In histological sections, hyperplasia of goblet cells and submucosal glands was not observed. For *Pseudomonas aeruginosa* challenge, no hamster developed fever after 72 hours and there was no mortality at the end of 14 days.

The results of this study show that the administration of enalapril or losartan did not have a significant effect on the expression of FOXA2, MUC5AC and MUC5B. Furthermore, the administration of enalapril or losartan did not cause the hamsters to develop hyperplasia of the goblet cells or submucosal glands. Finally, the administration of enalapril or Losartan did not cause the hamsters to develop fever. The implication is that enalapril and Losartan did not have a negative effect on the tracheal mucosal barrier function to *Pseudomonas aeruginosa* infection at a dose of 3.0×10^7 cfu/ml. In general, the findings of this study show that administration of enalapril or losartan does not significantly increase the production of respiratory tract mucins. Therefore, the administration of these drugs will not reduce the innate immune response of the respiratory tract through the impairment of mucus production and thus will not increase the susceptibility of individuals to *Pseudomonas aeruginosa* infection.

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PUBLICATIONS

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ABBREVIATIONS AND ACRONYMS

ACE	angiotensin converting enzyme.
ACEIs	Angiotensin converting enzyme inhibitors.
Ang II-	Angiotensin II
ANOVA	Analysis of variance
APC	Antigen presenting cell.
ARBs	Angiotensin receptor blockers
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
EGFR	epidermal growth factor receptor
FOXA2	Forkhead Box A2
GCH	Goblet cell hyperplasia
IL	Interleukin
MCP	Monocyte chemotactic protein
MHC	Major Histocompatibility complex
MUC5AC	Mucin 5AC
MUC5B	Mucin B
RAAS	Renin angiotensin aldosterone system
SARS-CoV 2	severe acute respiratory syndrome coronavirus 2
STAT6	Signal transducer and activator of transcription 6

CHAPTER ONE: INTRODUCTION

1.1 Background

The advent of Coronavirus disease 2019 (COVID-19) has brought with it new challenges, especially for people with existing respiratory and cardiac diseases related to the issue of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) (Richardson et al., 2020). ARBs and ACEIs are widely used in patients with hypertension and other cardiovascular diseases to improve the quality of life of affected persons (Messerli et al., 2018). Studies have shown that severe cases of covid-19 were observed in patients with hypertension, renal disease, and diabetes mellitus (Richardson et al., 2020). There are controversies as to why this is the case, and the role of angiotensin receptor blockers and angiotensin converting enzyme inhibitors is not being ruled out as a contributing factor (South et al., 2020). The COVID-19 virus penetrates the host cell through angiotensin converting enzyme 2 (ACE2). Studies have found that the expression level of ACE2 in cells was reduced significantly after COVID-19 infection, which lead to the increased level of angiotensin II (Ang II) and the activation of renin-angiotensin-aldosterone system (RAAS), thereby over activating the Ang II type 1 receptor (AT1R) of lung cells to induce and aggravate lung injury (Gong et al., 2021). ARBs and ACEIs interfere with the renin-angiotensin aldosterone system in producing their therapeutic effect. A study by Oussalah et al. (2020) found that long-term therapy with ACEIs / ARBs was associated with deleterious effects among patients with severe COVID-19 with regard to their risk of developing acute kidney injury and acute respiratory failure. However, other studies found that ACEIs / ARBs treatment could result in a marginally lower death rate and less critical cases in patients with COVID-19 and hypertension (Yang et al., 2020, Hippisley-Cox., et al., 2020). Additionally, Zhang et al. (2020) found that there was no association between the use of ACEI/ARBs and the severity of COVID-19.

The primary function of the respiratory system is to carry out gaseous exchange in aerobic organisms. However, this function can sometimes be hijacked by infectious and toxic particles which could negatively affect gaseous exchange (Lewis et al 2019). To defend itself from these infections, the respiratory system employs a host of mechanisms that work together to either remove the infectious insult or kill it. The mechanism for removing particles from the respiratory system is mainly the mucociliary clearance system, intercellular apical junctional complexes that regulate paracellular permeability, and antimicrobial peptides secreted by airway epithelial cells; these form the epithelial barrier function of the airway tract (Ganesan, 2013). The impairment of one or more of these essential components of epithelial barrier function can increase susceptibility to infection and promote prolonged and exaggerated innate immune responses to environmental factors, including allergens and pathogens, resulting in chronic inflammation. The mucociliary clearance system includes two major functional mechanisms; these are, ciliary beating and mucous secretion systems, each of which is usually complimentary and cooperative (Umeki, & Manabe, 1992). Further, the mucociliary system is made possible because of the airway surface liquid (ASL). The ASL is a thin fluid layer that covers the airway surface at the interface between epithelial cells and air space (Widdicombe, 2002). The ASL is important in regulating airway pH, ciliary function and mucociliary activity. The airway surface liquid is made up of two layers, namely the mucus layer and the periciliary layer. The mucus layer is made up of gel-forming mucins Mucin-5ac (MUC5AC) and Mucin-5b (MUC5B) which trap and remove inhaled particles from the airway system. Mucus clearance is the primary defense mechanism that protects airways from inhaled infections and toxic agents (Button et al 2012). Mucus hyper-secretion negatively affects the mucociliary clearance system by increasing the viscosity of the mucus. Studies have shown that depletion of the Forkhead box protein A2

(FOXA2) leads to hyperplasia of mucin producing glands in the respiratory system thereby resulting to mucus hyper secretion (Choi et al 2019). FOXA2 regulates and maintains airway mucus homeostasis. Organisms like *Pseudomonas aeroginosa* and *Bordetella bronchosplica* produce compounds that cause the depletion of FOXA2 in the respiratory system (Choi et al 2020). FOXA2 is important in the normal homeostasis of the lung environment and its depletion leads to worsening of lung infections. No studies were found that show the effect of ARBs and ACEIs on respiratory mucins. Among the most recognized examples of adverse side effects of ACE inhibitors are cough and angioedema, both of which are associated with elevated levels of bradykinin in patients taking these drugs (Byrd et al., 2006). Bradykinin is elevated in these patients because inhibition of angiotensin converting enzyme (ACE) inadvertently means that bradykinin inactivation is down-regulated, thereby increasing bradykinin (Karamyan, 2021). The increase in bradykinin promotes vascular leakage and fluid extravasation; this, in turn, can lead to pulmonary oedema and inflammation. Angiotensin receptor blockers (ARBs) have also been shown to increase bradykinin. ARBs increase bradykinin by reducing the activity of neutral endopeptidase (Walther et al. (2002); neutral endopeptidase are important in the metabolism of bradykinin (Nowak et al., 2011).

This study aimed to investigate the effects of ACEIs and ARBs on inflammatory mediators and how the inflammatory mediators affect the barrier function of the hamster tracheal mucosa.

1.2 Problem statement

Acute respiratory infections, in particular pneumonia, remain one of the most important causes of death in both adults and children (Bosch et al., 2013). Disease conditions that impact the consistency of mucosal secretions, for example in cystic fibrosis (CF), make people to be susceptible to chronic lung infections, including infections from *Pseudomonas aeruginosa* (Ciofu et al., 2013, Lyczak et al., 2002). Lungs of CF patients are often colonized with organisms, such as *Staphylococcus aureus, Streptococcus pyogenes* and *Haemophilus influenzae*, that may damage the epithelial surfaces of the respiratory tract, leading to increased attachment of, and eventual replacement by, *P. aeruginosa* (Lyczak et al., 2002). Prevention of infection in the respiratory system is maintained by the respiratory epithelium barrier function, consisting, mucociliary clearance system, intercellular apical junctional complexes that regulate paracellular permeability, and antimicrobial peptides secreted by airway epithelial cells (Ganesan, 2013). The impairment of one or more of these essential components of barrier function can increase susceptibility to infection and promote prolonged and exaggerated innate immune responses to environmental factors, including allergens and pathogens (Yuksel and Turkeli, 2017). The advent of COVID-19 has brought new challenges, especially for people with existing respiratory and cardiac diseases. The use of angiotensin receptor blockers and angiotensin converting enzyme inhibitors has been suspected to contribute to the severity of COVID-19, because they are associated with cough as one of the major side effects. Furthermore, secondary infections of bacterial origin might be significant in complicating COVID-19 disease progression. Severe cases of COVID-19 were observed in patients with hypertension, renal, and diabetes mellitus diseases (Richardson et al., 2020). There are controversies as to why this is the case, and the role

of angiotensin receptor blockers and angiotensin enzyme inhibitors is not being ruled out as a contributing factor (South et al., 2020). Although ARBs and ACEIs are associated with a dose dependent induction of cough as their side effect, no studies were found that show the effect of ARBs and ACEIs on Mucin-5ac, Mucin-5b and Forkhead box protein A2 of the respiratory system, especially in the wake of COVID-19 pandemic. Excessive production of mucus in the respiratory tract can be one of the causes of coughing. Mucin-5ac and Mucin-5b are major matrix forming mucins in the respiratory system and the viscoelasticity properties of mucus depend on them. Changes to the levels of these mucins will affect the mucociliary transport; Increased production of mucins will lead to thick mucus that impairs mucociliary transport (Shen et al., 2018). Forkhead box protein A2 is important in regulating and maintaining airway mucus homeostasis, and its depletion may lead to mucus hypersecretion and hyperplasia of mucus producing glands (Choi et al 2019).

1.3 Study justification

The study produced results that highlight the effects of ARBs and ACEIs on the respiratory mucins. Furthermore, the results of this study provided insights to the effects of ACEIs and ARBs on the mucosal barrier functions of the respiratory system, especially the effects pertaining to mucociliary escalator. In addition, the study provided some answers to the controversies pertaining to the effect of ARBs and ARBs on respiratory innate immunity. Furthermore, the study used a Syrian hamster as an animal model. The choice of the hamster was because the immune responses of the hamster to infectious pathogens are similar to those of humans (Miao et al., 2019; Iwatsuki-Horimoto et al., 2018) and as such, this model is advantageous for studying the pathogenesis of infections, assessing the efficacy and interactions of medications and vaccines for pathogens (Miao et al., 2019).

1.5 Hypothesis

Oral administration of angiotensin converting enzyme inhibitors and angiotensin receptor blockers impairs the innate immune response of the trachea by increasing the production and viscosity of mucus thereby increasing the susceptibility of individuals taking ARBs and ACEIs to secondary infections such as *Pseudomonas aeruginosa* infections.

1.6 General study objective

To study the effects of angiotensin receptor blockers and angiotensin converting enzyme inhibitors on the tracheal mucosal epithelial barriers to *Pseudomonas aeruginosa* infection.

1.6.1 Specific objectives were to:

1**.** Compare serum bradykinin in hamsters that have been treated with ACEIs or ARBs to those that have not been treated.

2. Compare goblet cell hyperplasia, inflammation and any changes in mucosal cells and submucosal cells for treated and untreated hamsters.

3. Compare the expressions of FOXA2, MUC5AC and MUC5B in the trachea of hamsters treated with ACEIs or ARBs versus those that have not been treated.

4. Compare the establishment of severe *Pseudomonas aeruginosa* infection in hamsters treated with ARBs/ACEIs and the control.

CHAPTER TWO: LITERATURE REVIEW

2.0 An overview of ACEIs, ARBs and COVID-19

Angiotensin receptor blockers and angiotensin converting enzyme inhibitors are widely used in patients with hypertension and other cardiovascular diseases to improve the quality of life of affected people. (Messerli et al., 2018). ACEIs are medications used in the treatment and management of hypertension, cardiac failure, myocardial infarction, in secondary prevention after myocardial infarction, and for kidney protection in diabetic and non-diabetic nephropathy (Aronson, 2016 and Herman et al., 2020). ACEIs differ in the chemical structure of their active groups, in potency, in bioavailability, in plasma half-life, in route of elimination and thus, the side effects of ACEIs can be divided into those that are class specific and those that relate to specific agents. ACEIs can be classified into three broad groups based on chemical structure:

1. Sulfhydryl-containing ACEIs structurally related to captopril 2. Dicarboxyl-containing ACEIs structurally related to enalapril (e.g., lisinopril, benazepril, quinapril, moexipril, ramipril, trandolapril and perindopril. 3. Phosphorus-containing ACEIs structurally related to fosinopril. Class specific side effects of ACEIs include hypotension, cough, hyperkalemia, renal failure, fetal anomalies, angioedema and dysgeusia, while Sulfhydryl-related effects are neutropenia, rash and nephrotic-range proteinuria (Brown & Vaughan, 1998).

ACEIs interfere with the renin-angiotensin-aldosterone system (Hanif et al., 2010). They competitively inhibit the angiotensin-converting enzyme to prevent the conversion of angiotensin I to angiotensin II (Miller and Arnold, 2019). Decreased production of angiotensin II improves natriuresis, lowers blood pressure, and prevents remodeling of smooth muscles and cardiac myocytes (Herman, 2020). ACEIs also interfere with the degradation of bradykinin, a peptide

that causes vasodilation (Karamyan, 2021). Inhibition of the renin–angiotensin system by ACE inhibitors has been shown to be effective in the treatment of hypertension, cardiac failure, myocardial infarction, in secondary prevention after myocardial infarction, and for kidney protection in diabetic and non-diabetic nephropathy (Aronson, 2016). Some examples of ACEIs that are used in Zambia include, enalapril, captopril, and lisnopril (Zambia essential medicine list, 2017).

Enalapril is one of the most prescribed angiotensin converting enzyme inhibitors (Okumu, 2018). It is used in the management of hypertension and congestive heart failure (faruqi, 2021). Enalapril is absorbed rapidly when given orally and has an oral bioavailability of about 60% and food does not affect the absorption and metabolism of enalapril (Brunton et al., 2018). Some of the complications that arises from taking enalapril are cough 5%–20% (Brunton et al., 2018) and angioedema 0.68% (Kostis et al., 2005) both of which are associated with elevated levels of bradykinin and substance-p (Byrd et al., 2006). The increase in bradykinin promotes vascular leakage and fluid extravasation, which, in turn, can lead to pulmonary angioedema and inflammation in the respiratory tract (Caballero et al., 2011).

A case of acute respiratory distress, secondary to severe angioedema, was recorded in an African-Caribbean male in his sixties who was admitted to a City Hospital, Birmingham, after taking enalapril 2.5mg for five months (Gibbs et al., 1999). In addition, two cases of angioedema were recorded in a Rural Health facility in Kenya; the cases were of a 55-year-old male and a 58-year-old woman who were taking 5mg of enalapril once per day (Okumu, 2018). Patients taking enalapril may develop angioedema within 14 days after taking the drug, and in 70% of the cases angioedema may develop after taking enalapril for four weeks (Gibbs et al., 1999).

Angiotensin receptor blockers (ARBs) were developed to overcome the deficiencies of ACEIs. For example, the risk of developing angioedema is lower with ARBs than with ACEIs. In fact, ACEIs are associated with an approximately 3-fold higher risk of angioedema (Toh et al., 2013). ARBs mechanism of action is also different from ACEIs in that ARBs block the effects of angiotensin II in producing its blood pressure lowering effects (Barreras and Turner, 2003), while ACEIs block the production of angiotensin II. ARBs bind to the angiotensin II angiotensin 1 receptor (AT1 receptor) thereby inhibiting the multiple actions of angiotensin II that are mediated by that receptor, including vasoconstriction, mitogenic activity, cytokine production, reactive oxygen species formation, and aldosterone production (Taylor et al., 2011). There are pharmacological differences among the various ARBs; for example, certain drugs in the class compete with angiotensin II in a concentration-dependent manner for binding to the AT_1 receptor, these are called surmountable antagonists examples include, Eprosartan, Valsartan and Azilsartan, while others are insurmountable antagonists that bind irreversibly to the receptor example include Candesartan, Irbesartan, Telmisartan, and Losartan (Oparil, 2000; Taylor et al., 2011). ARBs are generally well tolerated and have a low incidence of side effects. The side effects of ARBs are angioedema 0.1-0.2% (Taylor et al., 2011), hypotension, dizziness, dry cough, loss of taste, skin rash and kidney damage (Brunton et al., 2018). Losartan is the only ARB listed as an essential drug in Zambia (Zambia essential medicine list 2017). Losartan is an angiotensin II receptor blocker which binds to the AT1 receptor thereby blocking angiotensin II induced physiological effects (Siegl et al., 1995). Losartan can be administered without regard to food and approximately 14% of Oral dose of losartan is converted to the pharmacologically active E3174 metabolite in the liver. E3174 is 10 to 40-fold more potent than its parent compound and its estimated terminal half-life ranges from 6 to 9 hours (Sica et al., 2005). The

most common side effects of losartan include hyperkalemia, renal insufficiency, and hypotension (Bolotova et al., 2020). In a study by Goldberg et al. (1995) the most commonly reported clinical adverse experiences in patients treated with losartan were, headache (14.1%), upper respiratory infection (6.5%), dizziness (4.1%), asthenia/fatigue (3.8%) and coughing (3.1%). Losartan is approved in many countries for the treatment of hypertension and heart failure (Moen & Wagstaff, 2005). Losartan like Enalapril may be associated with angioedema. (Cha & Pearson, 1999; Nair, 2010; Orgaz-Molina et al., 2011). ARBs may cause angioedema by elevated levels of bradykinin (Byrd et al., 2006). A study by Campbell et al. (2005) found that losartan increased bradykinin levels by twofold in hypertensive humans. Furthermore, a study by Walther et al. (2002) showed that ARBs increase bradykinin levels by reducing the activity of neutral endopeptidase; neutral endopeptidase are important in the metabolism of bradykinin (Nowak et al., 2011).

The advent of COVID 19 has brought some controversies as to what role ARBs and ACEIs have on the severity of COVID. Studies have shown that patients with hypertension, renal, and diabetes mellitus diseases and taking ARBs or ACEIs have severe cases of COVID 19 (Richardson et al., 2020). However, a study by Lopes et al. (2021), found that, there was no significant difference between patients hospitalized with mild to moderate COVID-19 who took ACEIs or ARBs before hospital admission and those who discontinued the use of ACEIs or ARBs at the time of admission. Moreover, a study by Hippisley-Cox et al. (2020) found that ACEIs and ARBs were associated with reduced risks of COVID-19 disease. Additionally, a study by Xie et al. (2022) found that patients treated with ACEIs, or ARBs had a reduced risk of severe/death outcomes in covid-19, especially in Asia. Furthermore, a study by Li and Sarangarajan et al. (2021) found that in-hospital use of ARBs was associated with a significant

reduction in in-hospital mortality among COVID-19-positive African American patients. Similarly, a study by Armstrong et al. (2021) found that use of ARBs/ACEIs was associated with a decreased risk of being diagnosed with COVID-19. On top of that, a study by Lee et al. (2020) found that the use of ACEIs or ARBs does not increase angiotensin converting enzyme 2 (ACE2) expression in the upper respiratory cilia and therefore patients taking ACEIs, or ARBs are likely to have no higher risk of SARS-CoV-2 transmission than individuals not on these medications. Angiotensin converting enzyme 2 (ACE2) has been established as the functional host receptor for SARS-CoV-2, the virus responsible for the current COVID-19 pandemic (Bourgonje, 2020. However, a study by Li et al. (2021) showed that among COVID-19 positive Veterans with hypertension, the use of ACEIs was associated with increased odds of hospitalization. Furthermote, a study by Najafi et al. (2023) concluded that ARBs and ACEIs put COVID-19 patients at high risk for moderate to severe forms of COVID-19 and higher length of hospital stay. It should be noted that there was no significant association between the dose of ACEIs or ARBs and COVID-19 infection or hospitalization (Dublin et al., 2021). Conflicting results in observational studies raise the question whether antihypertensive classes have different effects on the outcome of COVID19 (Agirbasli, 2022). The interpretation of COVID19 outcomes and the use of ARBs/ACEIs requires caution, because older patients with multiple comorbidities and cardiovascular disease are also the group that are most likely to use ACEIs/ARBs. Older age is also a risk factor for server COVID19 (Abul et al., 2023; Singhal et al., 2021), the same age group which is likely to use ARBs/ACEI; for this reason, studies that do not adjust for age and other possible confounding factors may give wrong conclusions about their results (Agirbasli, 2022). Most of the evidence so far indicate that ACEIs/ARBs may have some protective effects during COVID19 infection if we adjust for age (Hippisley-Cox et al., 2020; ElAbd et al., 2021).

However, the question that begs an answer is are co-morbidities associated with COVID19 outcome? Or do ARBs/ACEIs differ in their class effects on COVID19 outcome? Further studies are required to conclusively state what effect ARBs /ACEIs may have during COVID19 infection. Physiologically, ACE2 plays a role in the regulation of three systems that could potentially be involved in the pathogenesis of severe COVID-19: the kinin-kallikrein system (KKS), the renin-angiotensin aldosterone system (RAAS), and the coagulation system (Sidarta-Oliveira et al., 2020), which systems the ARBs and ACEIs interfere with for their therapeutic outcomes in the management of cardiovascular diseases and renal diseases. SARS-CoV-2-ACE2 interaction led to the concerns about the use of ACE inhibitors and ARBs in infected patients with Sars-CoV-ACE2 (Agirbasli, 2022). ACE2 attenuates des-Arg^{9} bradykinin (DABK) mediated inflammation via Bradykinin 1 receptor activation by degrading DABK to BK (Sodhi et al., 2018). Therefore, the interactions between ACE, ACE2 and RAAS are critical in controlling the KKS in a state of excessive inflammation such as COVID19. Angiotensin converting enzyme 2 (ACE2) is the receptor for SARS-CoV-2. Binding occurs through viral spike protein (Sidarta-Oliveira et al., 2020), and depends on the transmembrane serine protease 2 **(**TMPRSS2) for priming (Hoffmann et al., 2020). Upon SARS-CoV-2 binding, ACE2 is internalized to endosomes, leading to a subcellular location shift that could alter its capacity to physiologically regulate the KKS and RAAS (Ou et al., 2020; Sidarta-Oliveira et al., 2020). After entry into the cell, SARS-CoV-2 initiates the production of new viruses (Lamers & Haagmans, 2022). After infection, the host will express type 1 interferon (T1IFN). Expression of T1IFN in the early stages of infection results immediately after the recognition of virions by pattern recognition receptors (PRRs) (Sharma et al., 2021). T1FN triggers the expression of interferon-stimulated genes (ISGs), which upregulate the effector function of immune cells (e.g.,

dendritic cells, B cells, and T cells) and generation of inflammatory cytokines and chemokines toward successful resolution of the infection (McNab et al., 2015). However, SARS-CoV-2 dysregulation of the immune system trigger multisystem inflammatory syndrome and evade host antiviral immune responses by downregulating early production of type I and type III interferons (IFN) and increase production of pro-inflammatory cytokine like interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) (Galani et al., 2021) ; the virus causes an abnormal activation of different cell types and persistent pro-inflammatory cytokine production. The ensuing hyperinflammatory state is believed to induce edema, fibrosis, and thrombosis in the lungs leading to hypoxia, acute respiratory distress syndrome (ARDS), and death in severe cases of the disease (Kudryavtsev et al., 2022). For instance, 'cytokine storm', induces extrapulmonary involvement and possibly fatal complications associated with multisystem inflammatory syndrome (Xu et al., 2022). The pro-inflammatory state stimulates uncontrolled complement activation and development of neutrophil extracellular traps (NETS), both of which promote the coagulation cascade and induce a state of microvascular injury and thrombosis of large and small blood vessels (Borczuk & Yantiss, 2022). Immune dysregulation by the SARS-CoV-2 may explain the symptoms observed during the COVID-19. Despite the overflow of research on SARS-CoV-2 pathogenesis, published information in this area may not completely explain the pathogenesis of COVID19, thus further research is needed.

2.1 Mucosal epithelial barrier

The function of gas exchange for the respiratory system exposes it to foreign particles, including infectious agents, allergens, and other substances that can damage it. The purpose of airway mucosa is to function as a physical barrier between the external environment and internal milieu

(Hewitt & Lloyd, 2021). The respiratory system mucosal barrier function is composed of mucociliary escalator, intercellular apical junctional complexes (Tight Junctions and Adherens junction) that regulate paracellular permeability, and antimicrobial peptides secreted by airway epithelial cells (Ganesan et al., 2013). The three components work together to remove inhaled pathogens, allergens, and particles without inducing inflammation and maintain tissue homeostasis; therefore, impairment of one or more of these components may increase the susceptibility of the respiratory system to infections and may prolong innate immune responses to environmental factors that can result in chronic inflammation (LeMessurier et al., 2020; Yuksel & Turkeli ,2017; Ganesan et al., 2013).

Mucociliary transport is composed of mucus and cilia; mucus traps inhaled pathogens and other particulate material, while coordinated ciliary beating sweeps the trapped material away from the lungs toward the pharynx (Hewitt & Lloyd, 2021). The airway mucus contains more than 200 proteins and is secreted by both goblet cells and submucosal glands (Ganesan et al., 2013). The major macromolecular constituents of the mucous layer are the gel-forming mucins MUC5AC, MUC5B, MUC2, MUC8, and MUC19 (Bustamante-Marin & Ostrowski, 2017). In healthy airways, the goblet cells typically express MUC5AC, whereas mucosal cells of the submucosal glands express primarily MUC5B (Groneberg et al., 2002). MUC5AC and MUC5B are the major matrix-forming macromolecules in airway mucus, and the viscoelastic properties of airway mucus depend on them (Bonser & Erle 2017). Increased production and secretion of MUC5B and MUC5AC can lead to thick mucus and impairment of the mucociliary escalator (Rose & Voynow, 2006; Bonser et al., 2016).

Antimicrobial peptides (AMPs) are expressed in the respiratory tract and act as effector substances of the innate immune system (Beisswenger & Bals 2005). Antimicrobial peptides are

present in airway surface fluid (ASF); the mucin-rich fluid covering the respiratory epithelium (Laube et al., 2006). Some of the antimicrobial peptides include, lysozymes, lactoferrin, cationic defensin, cathelicidin peptides, MUC5AC, MUC5B, human beta-defensin (hBD)-1, Bactericidal Permeability-Increasing protein, and Lactoperoxidase (Sibila et al., 2019; Laube et al., 2006; Rogan et al., 2006). AMPs are involved in opsonization facilitating phagocytosis of bacteria and viruses by macrophages and monocytes, act as mediators in inflammatory pathways and are capable of binding bacterial endotoxins (Rogan et al., 2006). In addition, AMP functions through protein degradation (lysozyme), nutrient depletion (lactoferrin), cellular disruption and lysis, and inhibition of virulence factors (Secretory leukocyte protease inhibitor) (Sibila et al., 2019). Intercellular apical junctional complexes that regulate paracellular permeability consist of tight Junctions and adherens junctions**.** The integrity of respiratory epithelium, fluid balance, and transport of molecules is dictated by the tight junctions and Adherens junction formed between adjacent cells (Niessen, 2007; Godbole et al., 2022). Epithelial cells are connected via tight junction (TJ) proteins on their apical surface at the air–liquid interface (Godbole et al., 2022). Tight junctions consist of the transmembrane proteins occludin and claudin, and the cytoplasmic scaffolding proteins zonula occludens 1, zonula occludens 2 and zonula occludens 3 (Hartsock & Nelson, 2008). Tight junctions regulate the paracellular pathway for the movement of ions and solutes in-between cells. Adherens junctions consist of the transmembrane protein E-cadherin, and intracellular components, p120-catenin, β-catenin and α-catenin (Hartsock & Nelson, 2008). Adherens junction is located right below tight junctions and provides an additional structural support and a strong bond between the sides of adjacent epithelial cell membranes (Niessen, 2007). The three barrier functions of airway epithelial tract, mucociliary clearance, intercellular

apical junctional complexes, and antimicrobial peptides of airway function together to effectively clear inhaled pathogens, allergens and pollutants from the respiratory system.

2.2 Role of angiotensin converting enzyme in the immune system

Angiotensin converting enzyme (ACE) is crucial in the renin-angiotensin-aldosterone system (RAAS) but is also involved in immune regulation (Oosthuizen and Sturrock, 2022). Immune regulation of ACE is via angiotensin II dependent effects and angiotensin II independent effects. Angiotensin II independent effects are effects in which immune regulation is based on the effects of ACE itself and not it products. In angiotensin II independent effects, angiotensin converting enzyme is involved in cleaving and altering the activity of macromolecules and cells of the immune system. For example, functional ACE is expressed in antigen-presenting cells (APCs), where ACE cleaves peptides and alters the class I repertoire of major histocompatibility complex (MHC) peptides (Shen et al., 2011). This repertoire is critical to CD8(+) T cell-mediated adaptive immune responses. Furthermore, ACE is also involved in cleaving C3, which is a complement protein (Semis et al., 2019). C3 is important in initiating complement system activation and plays a critical role in innate immune surveillance (Ricklin et al., 2016). In the same vein, increased expression of ACE by macrophages or neutrophils improves the ability of these cells to respond to immune challenges such as infection; however, administration of ACE inhibitors may reduce the ability of neutrophils and macrophages to kill bacteria (Cao et al., 2022). Finally, ACE inhibition can affect T lymphocyte activation and proliferation by altering the immune response through a change in cell surface signals (Petrov et al., 2000). Angiotensin II-dependent effects are those in which angiotensin II exerts immune regulation; the effects are based on the activity of angiotensin II. For example, angiotensin II (Ang II) promotes pro-inflammatory responses and macrophage activation *via* the angiotensin-1 receptor (AT_1R).

Furthermore, Ang II induces the expression of cytokines, including monocytic chemotactic protein (MCP-1), Interleukin-8 (IL-8) and interleukin-18 (IL-18), which are involved in macrophage recruitment and promote monocyte differentiation and polarization, thus directly enhancing phagocytosis. Drugs that affect the production of ang II or its actions will have a negative effect on macrophage recruitment and monocyte differentiation.

ACEIs competitively inhibit the angiotensin-converting enzyme to prevent the conversion of angiotensin I to angiotensin II (Miller and Arnold, 2019), whereas ARBs block the effects of angiotensin II in producing their blood pressure-lowering effects (Barreras and Turner, 2003). The action of both ARBs and ACEIs on the renin-angiotensin-aldosterone system has the potential to negatively affect the immune system. Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) were at the center of debate about their effect during COVID-19 infection (Richardson et al., 2020).

2.3 Role of FOXA2, MUC5AC, and MUC5B in innate immunity of the respiratory tract

The respiratory mucosal surface is continuously exposed to inhaled pathogens and a protective layer of secreted mucus acts as a first line of defense against infections (Button et al., 2012). In chronic inflammatory respiratory disorders, mucine-5B (MUC5B) and mucin-5AC (MUC5AC) increase (Silva and Bercik, 2012). A higher ratio of MUC5AC to MUC5B correlates with type 2 inflammation (Bustamante-Marin & Ostrowski, 2017; Lachowicz-Scroggins et al., 2016). MUC5B and MUC5AC are major matrix-forming macromolecules in airway mucus, because of this, viscoelastic properties of airway mucus depend on them (Bonser and Erle, 2017). The viscoelastic properties of mucus are important in mucociliary clearance. Mucociliary clearance (MCC) is the primary innate defense mechanism of the respiratory tract (Bustamante-Marin &

Ostrowski, 2017). Thick mucus can impede mucociliary transport and cause obstruction of the airways; this will in turn cause the respiratory system to fail at removing infectious agents and other particles. (Singanayagam et al., 2022).

In the baseline conditions of chronic inflammatory respiratory disorders, mucine-5B (MUC5B) and mucin-5AC (MUC5AC) increase in different compartments of the respiratory tract. Macrophages induce MUC5B expression in bronchiolar goblet cell hyperplasia, suggesting a role for them in the pathogenesis of airway MUC5B-related goblet cell hyperplasia (GCH) (Silva and Bercik, 2012). However, Seibel et al. (2018) stated that the release of MUC5AC from goblet cells is typically upregulated after inflammatory events in the airways through activation of the interleukin-4 (IL-4) /interleukin-13 (IL-13) receptor and epidermal growth factor receptor (EGFR) signaling. A higher ratio of MUC5AC to MUC5B correlated with type-2 respiratory inflammation (Lachowicz-Scroggins et al., 2016.

The Forkhead box protein A2 (FOXA2) is a key transcriptional regulator that maintains airway mucus homeostasis; as such, the changes that affect FOXA2 can cause changes in the properties of respiratory mucus. Depletion of FOXA2 results in goblet cell hyperplasia and metaplasia, leading to mucus hypersecretion (Choi et al., 2020; Choi, Choe and Lau, 2020). Mucus hypersecretion has been found to reduce drug penetration in the respiratory system, making it difficult to treat respiratory infections (Choi et al., 2020; Wan et al., 2004).

Organisms such as *Pseudomonas aeroginosa* and *Bordetella bronchiseptica* produce compounds that cause the depletion of FOXA2 (Choi et al., 2020). FOXA2 is important in the normal homeostasis of the lung environment and its depletion leads to worsening of lung infections. Factors such as pyocyanin that is produced by *Pseudomonas aeruginosa* have been shown to deplete FOXA2 via the EGFR and Signal transducer and activator of transcription 6 (STAT6)

pathway (Hao et al., 2012), while the pertussis toxin produced by *Bordetella bronchoseptica* is key to pulmonary oedema and pneumonia (Chebel et al., 2017).

2.4 Secondary Bacterial infections during Viral infections

Secondary bacterial infections occur during or after viral infections and can lead to adverse results and sometimes fatal clinical complications (Manohar et al., 2020). A study by Bahceci et al. (2022) found that 8.7% of COVID-19 patients had microbiologically proven secondary bacterial infections. In their study, Bahceci et al. (2022) found that *coagulase negative staphylococci, Acinetobacter baumannii,* Staphylococcus *aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa* were the most isolated secondary bacterial infections. Another study by De Bruyn et al. (2022) found that 68% of patients with COVID-19 acquired at least one of the secondary bacteria studied; the study also found that 65.96% acquired secondary bacterial pneumonia caused by gram negative bacili, while 29.79% acquired bacteremia of unknown origin and a smaller proportion of patients 14.89% acquired catheter-related sepsis. In addition, a study by Rouzé et al. (2022) found that 64% of SARS-COV 2 patients and 33% of the influenza-infected patients were also infected *with Pseudomonas aeruginosa.* Furthermore, a study by Shafran et al. (2021), found that COVID-19 patients had higher rates of secondary bacterial infections than influenza patients; additionally, secondary bacterial infection was associated with a higher risk of death in both patient groups. Manohar et al. (2020) found that, in almost all severe cases of SARS-CoV-2 infection, secondary bacterial infections were caused by *P. aeruginosa* and *S. aureus*; moreover, for all cases of viral pneumonia, a mortality rate of 10.9% was due to secondary bacterial infections. Dysregulation of antimicrobial peptides and mechanical disruption of airway epithelium and its barrier function due to viral-induced immune-mediated damage, and dysregulation of both, the innate and adaptive immune responses are

thought to promote the colonization by secondary bacterial infections. For example, Influenza virus can induce the production of type I interferons (IFNs) that results in reduced production of monocyte chemoattractant protein-1 (MCP-1), thereby decreasing the recruitment of macrophages/monocytes thus resulting in enhanced colonization of *Streptococcus pneumonie* and *Staphylococcus aureus* (Manna et al., 2020). Furthermore, other viruses like, *Respiratory syncytial virus* (RSV) induces the adherence of *S. pneumoniae*, *Pseudomonas aeruginosa*, and *Haemophilus influenza* to airway epithelial cells (Avadhanula et al., 2007). In addition, Influenza virus has also been shown to suppress production of interleukin-17 (IL-17)- and IL-22-associated antimicrobial peptides such as lipocalin 2, damage-associated molecular patterns (DAMPs) and Cathelicidin antimicrobial peptide (CAMP) (Robinson et al., 2014). Immunosuppression has been observed in some viruses for example the human immunodeficiency virus reduces $CD4^+$ T-cells that make the patient much more susceptible to develop bacterial infections (Vittor et al., 2014). SARS-CoV-2 is very much similar to the SARS-CoV (Manna et al., 2020), SARS-CoV patients has been reported to have secondary bacterial infections and confirmed the presence of different bacteria in various human samples, including *Escherichia coli, K. pneumoniae, P. aeruginosa,* and Methicillin-Resistant *S. aureus* (Tan et al., 2005). SARS-CoV infection downregulates IFN-α/β and cathepsin (Manna et al., 2020). The presence of cathepsins in the endo-lysosomal compartment permits direct interaction with and killing of bacteria and contribute to processing of bacterial antigens for presentation, an event necessary for the induction of antibacterial adaptive immune response and long-term immunity (Szulc-Dąbrowska et al., 2020); thus, downregulation of cathepsin production will negatively affect the ability of macrophages to digest engulfed pathogens through phagocytosis, thereby decreasing the ability of macrophages to remove pathogens. A study by He et al. (2021)

found that COVID19 patients had extremely depressed numbers of CD4+ and CD8+ T cells; additionally, the absolute numbers of total T lymphocytes, $CD4^+$ T cells, and $CD8^+$ T cells in severe patients were all significantly lower than those in patients with moderate disease. $CD4^+$T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, and cytotoxic T cells; while $CD8^+$ T cells can mount a response against pathogens by secreting cytokines and chemokines, and directly lysing infected cells: this innate response has been shown to be beneficial in controlling several types of bacterial infections (Berg & Forman, 2006). Viral infections avail bacterial infections by using multiple strategies, including providing a more susceptible site for adhesion, altering immune response, and invasive infection by cell and tissue damage.

2.5 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative, rod-shaped, aerobic, non-spore-forming bacteria that is ubiquitous in the environment where it can cause disease in a variety of hosts including plants, nematodes, insects, and mammals (Diggle & Whiteley, 2020; He et al., 2004). It is an opportunistic pathogen that sets upon the host when the normal immune defenses are disabled (Mulcahy et al., 2014). The bacteria are also a major problem in patients with cystic fibrosis, burn wounds, chronic wounds, chronic obstructive pulmonary disorder (COPD), nosocomial infections like ventilator-associated pneumonia, catheter-associated urinary tract infections, and Asthma (Jurado-Martín et al., 2021). Disease causation by *P. aeruginosa* is because of producing cell-associated or secreted compounds like pyocyanin that drive its virulence (Qin et al., 2022; Alonso et al., 2020). Pyocyanin, a blue greenish color metabolite of *P. aeruginosa* colonies in culture (Gellatly & Hancock, 2013) is associated with disease severity and decline in lung function due to pro-inflammatory effects (Hall et al., 2016). Pyocyanin causes accelerated

neutrophil apoptosis in the lungs resulting in impaired bacterial clearance; also, high pyocyanin production in vitro was found to be predictive of poor disease outcomes (Gupte et al., 2021). Furthermore, pyocyanin slows ciliary beating, causes epithelial disruption, and increases mucous secretion in the respiratory tract (Hall et al., 2016), contributing to lung infection. *Pseudomonas aeruginosa* genome is made up of a single circular chromosome and a variable number of plasmids (Klockgether et al., 2011). Plasmids are critical in bacterial fitness and evolution of *Pseudomonas aeruginosa*. For example, a study by Ramírez-Díaz, et al. (2011) found that a virulence plasmid was capable of conferring antibiotic resistance when it was transferred to a recipient strain of *P. aeruginosa*. Furthermore, another study by Amiel et al. (2010) found that loss of motility function harbored by plasmids in *P. aeruginosa* was associated with increased bacterial burdens and increased disease severity in cystic fibrosis patients. Studies have shown that *P. aeruginosa* can colonize and cause infection in mice (Facchini et al., 2014; Bachta et al., 2020) and in hamsters (Bhavsar et al., 2010; Coalson et al.,1986). *Pseudomonas aeruginosa* was selected for this study because it is an opportunistic pathogen and can cause a wide range of severe opportunistic infections in patients with underlying medical conditions (Gellatly & Hancock, 2013) but may not cause serious infection in immune-competent hosts. *Pseudomonas aeruginosa* PA1 used in this study is a virulent strain that was first isolated from a patient with respiratory tract infection in China (Lu et al., 2015).

2.6 Study Variables

From the published literature, no information was found that show what effect ARBs and ACEIs may have on the expression of respiratory FOXA2, MUC5AC and MUC5B; further no information was found documenting what effect taking ACEIs and ARBs may have in

Pseudomonas aeruginosa infection. FOXA2, MUC5AC and MUC5B are important in mucociliary transport which is important in epithelial barrier function.

In this study, Enalapril and Losartan represented ACEIs and ARBs respectively. Both drugs were administered orally. The choice of these drugs was because they are commonly used in Zambia and are listed as essential drugs in the list of essential medicines in Zambia. They are among the most prescribed drugs in the management of hypertension and cardiovascular disease (Okumu, 2018, Burnier & Wuerzner, 2011 and Arivazhahan et al., 2020). The study also used *Pseudomonas aeruginosa* PA1 as a bacterium to test the effect of ARBs and AECIs on the respiratory immune system of hamsters. *Pseudomonas aeruginosa* PA1 was selected because it is an opportunistic pathogen (Gellatly & Hancock, 2013).

This study also used a Syrian hamster as an animal model. The choice of the hamster was because the immune responses of the hamster to infectious pathogens are similar to those of humans (Miao et al., 2019; Iwatsuki-Horimoto et al., 2018) and as such, this model is advantageous for studying the pathogenesis of infections, including bacterial, viral, and parasitic pathogens, along with assessing the efficacy and interactions of medications and vaccines for these pathogens (Miao et al., 2019). In addition, the pathology of SARS-CoV2 in the Syrian hamster is similar to what is seen in humans (Sia et al., 2020, Suresh et al 2020). Furthermore, the expression of ACE2 in Syrian hamsters is similar to that in humans (Suresh et al., 2020); the similarities of ACE2 between humans and hamsters in this case means that the effect of ARBS and ACEIs in humans and hamsters is similar. Finally, the pulmonary vascular bed of the hamster is also similar to that of humans (Emily et al., 2015), which means that hamsters and humans may have similar pathogenesis of angioedema of the pulmonary system.

The study used bradykinin, MUC5AC, MUC5B, Goblet cell hyperplasia, changes in the tracheal submucosal gland, inflammation of the trachea epithelium, fever, and FOXA2 expression as response variables to measure the effect of ARBs and ACEIs on the tracheal barriers of the hamster Tracheal Mucosa.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Design

This was a randomized post-test only control group study design. The randomized post-test-only control group design is a basic experimental design where participants are randomly assigned to either receive an intervention or not, and then the outcome of interest is measured only once after the intervention takes place in order to determine its effect. The intervention can be: 1) a medical treatment, 2) a training program, and 3) an exposure to a risk factor, or other intervention. With this type of study design, no measurements are taken before the intervention (Shadish et al., 2001; Flannelly et al., 2018). The study used normal animals to investigate the effect of ACEIs and ARBs on tracheal mucosal barriers. In this study, normal health animals were used because the study aimed to investigate whether the use of ARBs and ACEIs can affect respiratory epithelial barriers and, consequently, to get some answers as to whether co-morbidities during COVID19 were responsible for the observed severe cases or did taking these drugs cause severe cases of COVID19. Three experiments were performed: Experiment 1 was to test the effect of ARBs and ACEIs on FOXA2, MUC5AC and MUC5B, these variables were tested because they affect the mucociliary escalator, which is one of the three barriers of the tracheal epithelial barriers. Experiment 2 was to test what effect the ARBs and ACEIs may have on the mucin producing glands (Goblet glands and Submucosal glands) and other histological changes to the tracheal mucosa of the hamster. Experiment 3 tested the effect of taking ARBs and ACEIs in *P. aeruginosa* infection.

3.2 Study Site

The study was conducted at the University of Zambia, School of Veterinary Medicine, Biochemistry, Histology and Microbiology laboratories.

3.3 Ethical Clearance

The ethical approval was granted by the University of Zambia Biomedical Research Ethics Committee (UNZABREC) IRB00001131 of IORG0000774. The approval number for the study is REF. No. 2504-2022.

3.4 Sample size

The sample population was calculated using the 'resource equation' method (Arifin & Zahiruddin, 2017). In this method, the degree of freedom of analysis of variance (ANOVA) (E) should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting a more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. $E = N - k = kn - k = k(n - 1)$, where **N** = total number of subjects**, k** = number of groups, and **n** = number of subjects per group. Rearranging the formula, n is given as: $n = E/k + 1$

a) Minimum sample size= $10/3 + 1 = 4.3$ rounding off = 4 animals per group
b) Maximum sample size=$20/3 + 1 = 7.7$ rounding off = 8 animals per group

The sample size per group was calculated to be between 4 and 8 animals per group. The average $(4 + 8) / 2 = 6$ animals per group were used in this study.

3.5 Drug Dose Calculation

The drugs used in this study were Enalapril maleate, which is an ACEI, and Losartan potassium, which is an ARB. The dose of the drugs was calculated from the maximum recommended dose for human treatment.

Enalapril: The maximum recommended dose is 40 mg per day (Cleary and Taylor, 1986).

- 40 mg per day translates to 0.58mg/kg/day taking 70 kg as maximum body weight to calculate the drug dosage (Pai, 2012).
- The water consumption of the Syrian male hamster is 5ml/100g body weight/day, and the female is 13.5 ml/100g body weight/ day (National Research Council (US), 1995).
- The mean weight of the Syrian male hamster is 100 g (Emily et al., 2015); furthermore, a male hamster drinks 5ml/100g body weight of water. Therefore, the dose of enalapril in drinking water to give an oral dose per day equivalent to 0.58 mg/kg/day is as follows: 0.58mg/1000g x 100g =0.058mg per day per hamster. Dissolved in water will be 0.058mg/5ml = 0.0116 ≈ 0.012mg/ml. converting this to mg/liter. 0.012mg/ml x 1000ml/L = 12mg/L. Male hamsters were given 12mg/L of Enalapril in drinking water for four weeks.

Losartan: The maximum recommended dose for losartan is 100 mg per day (Sica et al., 2005). Following the calculations done above, 100 mg per day translates to 1.4mg/kg/day in male hamsters. The dose of Losartan in drinking water was calculated to be 28 mg / L, which was equivalent to an oral dose of 1.4mg/kg/day.

Note: Dosage of drugs: Enalapril 12mg/L and Losartan 28mg/L in drinking water.

Experiment 1: Effects of ACEIs and ARBs on Bradykinin, MUC5AC, MUC5B and FOXA2

The objectives of this experiment were to 1) Compare the expression of FOXA2, MUC5AC and MUC5B in the trachea of hamsters treated with ACEIs or ARBs versus those that have not been treated and 2) compare serum bradykinin in hamsters that have been treated with ACEIs or ARBs to those that have not been treated. The hamsters were randomly put in three groups of six. The hamsters that were used for the study were moved from the common housing to a separate non-infectious room during the period of the experiment.

1. Group 1 was treated with distilled water and served as the control group.
2. Group 2 was treated with Enalapril 12mg/L.
3. Group 3 was treated with Losartan 28mg/L.

3.6 Animal treatment and control groups

For this study,18 male hamsters were used. Male hamsters were used to reduce confounders that may arise due to the oestrous cycle in female hamsters (Rabi et al., 2008). In addition, male hamsters were used to reduce the chances of using a pregnant hamster during the study. The hamsters were randomly separated into three (03) groups of six (06). A group of hamsters was equal to six hamsters for purposes of this study. The drugs that were used in the study were Enalapril Maleate USP 20mg manufactured by LIFEON LABS PVT, LTD India; Zambia Medicines Regulatory Authority (ZAMRA) reg no.201/028 and Losartan Potassium USP 50mg manufacture by Emcure Pharmaceuticals: ZAMRA reg no.195/010. One group of six (06) hamsters was treated with Enalapril 12mg/L in drinking water for 30 days, and another group of six (06) was treated with Losartan 28mg/L in drinking water for 30 days. The last set of six (06) of hamsters was given distilled water; this served as the control group. The drugs that were

administered to the hamsters were dissolved in distilled water. The dosage for the drugs was calculated from the maximum recommended dose in treating humans. For Enalapril, the maximum recommended dose is 40mg per day (Cleary and Taylor, 1986), while that of Losartan is 100mg per day (Sica et al., 2005). The hamsters had free access to food and water which contained drugs for the experimental animals and distilled water for the control animals. Distilled water was given to the control hamsters because the experimental hamsters received drugs dissolved in distilled water; thus, the only difference in drinking water between the experimental hamsters and the control was the dissolved drugs. The hamsters were feed with Nutrisure commercial pellets manufactured by NUTRIFeeds Zambia Limited, with a formulation of moisture content of 120g/kg-crude protein maximum, 13g/kg lysine maximum, 6.5g/kg-crude fat, 30/40g/kg-min/max crude fiber, 120g/kg-calcium, 10/12g/kg min/max and phosphorus-6g/kg-minimum. The hamsters were kept in cages with paper litter and the litter was changed twice every week during the study.

3.7 Sample Collection

Collection of blood to determine serum bradykinin.

Blood was collected by cardiac puncture using the diaphragmatic approach. General anesthesia (xylazine 10mg/kg body weight Payton et al.,1993) was administered to the animal until no pedal withdrawal reflexes were present. Cardiac puncture and blood collection was performed following a procedure by Beeton et al., (2007). The blood was put in a Becton Dickinson (BD) plain Vacutainer. The animals were sacrificed at the completion of the procedure prior to awakening from anesthesia. Blood was collected once from each hamster at the end of 30 days.

3.8 Removal of trachea

The trachea was removed from the sacrificed animal by cutting it from the larynx to the tracheal bifurcation following the procedure described by Parkinson et al., (2011). The trachea was removed by making an incision cranially and caudally along the ventral midline extending from the chin to the pubis. Blunt dissection was used to remove non-trachea associated tissue. The larynx was removed by making a transverse cut below the cricoid cartilage using a micro dissecting scissors. Further, the left and right primary bronchial branches were removed by making a transverse cut just above the carina using micro dissecting scissors. After removal, the trachea was cut into two approximately equal length pieces. One piece was put in 2.0 ml ice cold HIMEDIA phosphate buffer saline (PBS) PH 7.0; this piece was used to extract FOXA2, MUC5AC and MUC5B. The second piece was put in 10% formal saline; this piece was used for histology studies in experiment 2.

3.9 Extraction of FOXA2, MUC5AC and MUC5B.

The piece of tracheal tissue that was put in PBS was rinsed in fresh PBS and then it was homogenized in 1.0 ml ice-cold PBS. The homogenates were centrifuged for 5 minutes at 5,000 rpm, 4°C. The supernatant was removed, aliquoted in a 1.0 ml cryogenic via with screw cup and stored at -20°C and was assayed the following day. The aliquot used to measure the tracheal expression of FOXA2, MUC5AC and MUC5B using ELISA. The aliquots were centrifuged again after thawing before the assaying.

3.10 Extraction of Serum for bradykinin assay

After the blood clotted; serum was aliquoted from each vacutainer and separately put in 1.0 ml cryogenic vial and stored at -20°C. The vial was thawed at room temperature, before being essayed with ELISA.

Sample Analysis

3.11 Enzyme-Linked Immunoassay (ELISA) for Bradykinin.

The reagents for ELISA were prepared following the standard procedure by Wuhan Fine Biotech Co., Ltd reagents kit Manual for Hamster Bradykinin ELISA kit.

A micropipette was used to add 100ul of serum to the test sample wells, the plate was then sealed with adhesive strip and incubated for 90 minutes at 37ºC. After 90 minutes, the plates were taken out of the incubator, the adhesive strip was removed, and the plated contents were aspirated and discarded. The plate was washed using a multi-channel pipette 2.0 times with 200ul wash buffer. After washing, 100ul of diluted Biotin-labelled antibody (Biotin-labelled antibody dilution 1:100) was added to the bottom of each well without touching the side wall. The plate was covered with a new strip of adhesive and put in the incubator at 37ºC for 60 minutes. After 60 minutes, the contents of the wells were aspirated and discarded; the wells were washed using a multi-channel pipette 3.0 times with 200ul wash buffer, and the wash buffer was allowed to stay in the wells for 2.0 minutes each time. After washing, 100ul of diluted HRP-Streptavidin conjugate (HRP-Streptavidin conjugate dilution 1:100) was added to the wells, the plate was covered with adhesive strip and incubated at 37ºC for 30 minutes. After 30 minutes, the cover strip was removed, and the wells were washed for 5.0 times with wash buffer while

allowing the wash buffer to stay in the wells for 2.0 minutes each time. After washing 90ul of TMB substrate was added to each well, an adhesive cover strip was put on the plate and the plate was incubated for 20 minutes at 37^{o}C without light. Thereafter, 50ul of stop solution was added to each well. OD measurements were done at absorbance of 450nm using a microplate reader, Tecan Austria GmbH, 5082 Grödig, Austria Model SUNRISE BASIC TECAN serial number 1001004866. GraphPad Prism software version 10 was used to generate a standard curve and interpolate the unknown concentrations from absorbance readings.

3.12 Enzyme-Linked Immunoassay (ELISA) for FOXA2, MUC5AC AND MUC5B.

The reagents for ELISA were prepared following the standard procedure by Wuhan Fine Biotech Co., Ltd Hamster FOXA2, MUC5AC and MUC5B reagents kit manuals. Thereafter, 100ul of tracheal tissue extract was added to the test sample wells, the plate was then sealed with adhesive strip and incubated for 90 minutes at 37^{o}C. After 90 minutes, the plates were taken out of the incubator, the adhesive strip was removed, and the plated contents were aspirated and discarded. The plate was washed using a multi-channel pipette for 2.0 times with 200ul wash buffer. After washing, 100ul of diluted Biotin-labelled antibody (Biotin-labelled antibody dilution 1:100) was added to the bottom of each well without touching the side wall. The plate was covered with a strip of adhesive and put in the incubator at 37^{o}C for 60 minutes. After 60 minutes, the contents of the wells were aspirated and discarded, the wells were washed using a multi-channel pipette for 3.0 times with 200ul wash buffer, and the wash buffer was allowed to stay in the wells for 2.0 minutes each time. After washing, 100ul of diluted HRP-Streptavidin conjugate (HRP-Streptavidin conjugate dilution 1:100) was added to the wells, the plate was covered with adhesive strip and incubated at 37^{o}C for 30 minutes. After 30 minutes, the cover strip was removed, and the wells were washed for 5.0 times with 200ul wash buffer while allowing the

wash buffer to stay in the wells for 2.0 minutes each time. After washing 90ul of TMB substrate was added to each well, an adhesive cover strip was put on the plate and the plate was incubated for 20 minutes at 37ºC without light. To stop the reaction, 50ul of stop solution was added to each well. Optical Density measurements were done at absorbance of 450nm using a microplate reader, Tecan Austria GmbH, 5082 Grödig, Austria Model SUNRISE BASIC TECAN serial number 1001004866. GraphPad Prism software version 10 was used to generate a standard curve and interpolate the unknown concentrations of FOXA2, MUC5B and MUC5AC from absorbance readings.

Experiment 2: Effects of ACEIs and ARBs on Histology of Tracheal Mucosa

The objective of experiment 2 was to compare goblet cell and submucosal glands hyperplasia, inflammation and any changes to the mucosal cells and submucosal cells of the trachea. The tracheal tissue which was collected in experiment 1 and put in 10% normal formal saline was used in this experiment.

Sample analysis

3.13 Histopathology of Trachea

Tissue processing and paraffin embedding were done following a standard procedure by Dey, (2018). An 18-hour automatic tissue processing Schedule (Start 14:00 hours and ending at 08:00 hours) was followed.

18-hour automatic tissue processing Schedule below (Start 14:00 hours, End: 08:00 hours)

Bath No.	Fluid	Purpose	Time
1	10% Formal saline	Completion of Fixation	1 hour
2	70% Ethanol	Dehydration	1 hour
3	80% Ethanol	Dehydration	1 hour
4	95% Ethanol	Dehydration	1 hour
5	Absolute Ethanol (I)	Dehydration	1 hour
6	Absolute Ethanol (II)	Dehydration	1 hour
7	Absolute Ethanol (III)	Dehydration	2 hours
8	Xylene	Clearing	1 hour
9	Xylene	Clearing	1 hour
10	Xylene	Clearing	2 hours
11	Paraffin wax (molten) I	Impregnation	3 hours
12	Paraffin wax (molten) II	Impregnation	3 hours

3.13.1 Tissue Embedding

The tissue cassette containing the trachea was opened. The tissue was put in an embedding mould that contained molten paraffin wax using a warm pair of forceps. The tissue was placed such that transverse sections were cut during sectioning with a microtome. Some amount of pressure was applied on the tissue to make an even embedding. The mould was chilled on the cold plate, then the tissue was oriented and firmed into the wax with a warmed pair of forceps. This was done to ensure that the correct orientation was maintained and the tissue surface to be sectioned was kept flat. A labelled embedding cassette base was placed onto the mould, then more molten paraffin wax was put into the mould to fill the cassette and mould. The mould was cooled on the cold plate. Finally, the mould was removed from the block.

3.13.2 Tissue Sectioning

Excess wax was removed from the surface of the block. The blocks were chilled on a refrigerated cold plate for 10 minutes before sectioning. A microtome was used to slice thin tissue sections off the block in the form of a ribbon (5 µm). Once cut, the tissue ribbons were carefully transferred to a warm water bath. Here they were allowed to float on the surface and then were fished up onto a slide placed under the water level. The labelled slides were then allowed to dry upright at 37ºC for one hour to gently melt the excess paraffin wax. The slides were then put on a slide rack.

3.13.3 Tissue staining for histopathology.

Deparaffinization

Before proceeding with the staining protocol, the slides were deparaffinized and rehydrated. The slides were placed in a rack, the following washes were performed to remove the wax:

The slides were put in xylene two times for 2 minutes each time. Then the slides were moved to xylene 1:1 with absolute ethanol for 2 minutes. From xylene 1:1 absolute ethanol, the slides were placed in absolute ethanol two times for 2 minutes each time. Then the slides were put in 95% ethanol for 2 minutes, followed by 2 minutes in 70% ethanol and finally in 50% ethanol for 2 minutes. Finally, the slides were rinsed in cold tap water. At no time were slides allowed to dry during the deparaffinization process.

Periodic Acid Schiff (PAS)

After deparaffinization, the sections were rinsed in distilled water, and then oxidized in 1% period acid for 5 minutes. After 5 minutes, the sections were removed from periodic acid and

rinsed in distilled water and then washed in running tap water for 5 minutes. The sections were then rinsed in distilled water again before being treated with Schiff's reagent for 15 minutes. After 15 minutes, the sections were removed from Schiff's reagent and rinsed for 5 minutes in running tap water; then the sections were stained in Mayer's hematoxylin for 1 minute. The sections were then removed from hematoxylin and bluing was done in tap water for 5 minutes. Finally, the sections were rinsed in distilled water, then dehydrated in alcohol, cleared in xylene and mounted in DPX.

Haematoxylin and Eosin stain

After deparaffinization, the sections were stained with Mayer's hematoxylin for 10 minutes; thereafter, the sections were washed in running tap water for 5 minutes.

The sections were differentiated in 1% acid alcohol for 30 seconds. The sections were then blued in running tap water for 5 minutes and then counter stained in 1% aqueous eosin for 5 minutes. After five minutes the sections were rinsed in tap water by dipping the slide in the water for ten times. The sections were then dehydrated in 2 changes of absolute alcohol for 2 minutes in each, cleared in 2 changes of xylene for 2 minutes each and then mounted in DPX.

Experiment 3: Effects of ACEIs and ARBs on the tracheal mucosa barriers to *Pseudomonas aeruginosa* infection.

The objective of this experiment was to compare the establishment of severe *Pseudomonas aeruginosa* infection in hamsters treated with ARBs or ACEIs and the control. Before infecting the hamsters with *Pseudomonas aeruginosa*. The bacterial patient isolation was confirmed by sequencing. Further, before the hamsters were allocated to the groups nasal swabs were cultured and only those hamsters that were negative for *P. aeruginosa* were used for the study. The hamsters that were used for the study were moved to separate non-infectious housing for the duration of the study. Preliminary infectious dose for *P. aeruginosa* was established at 3.0×10^7 cfu/ml. The hamsters were separated into four groups of six. The first group was treated with enalapril, the second group was treated with losartan, the third group was treated with distilled water and the fourth group was treated with distilled water as well. The dosage of the drugs and duration was the same as in experiment 1.

Experimental design was as follows:

a) Group 1: six distilled water treated hamsters + *P. aeruginosa.*

b) Group 2: six enalapril treated hamsters + *P. aeruginosa.*

c) Group 3: six losartan treated hamsters + *P. aeruginosa.*

d) Group 4: six distilled water treated hamsters + phosphate buffered saline.

Sample analysis

3.14.0 Pseudomonas *aeruginosa* infection of hamsters

3.14.1 Source of Pseudomonas aeruginosa Isolate used in the study.

An Isolate of *Pseudomonas aeruginosa* collected from a patient stored in 20% glycerol at -80 ^{o}C was obtained from Tropical Diseases Research Centre (TDRC) Zambia, under strict microbial containment. The vial containing the bacteria was allowed to thaw at room temperature in the laboratory. The isolate was then sub-cultured on nutrient agar and incubated aerobically at 37^{o}C for 24 hours. Before proceeding, the isolate was reconfirmed as *Pseudomonas aeruginosa* using molecular methods.

3.14.2 Molecular reconfirmation of Pseudomonas aeruginosa strain.

DNA extraction

After 24 hours of growth on Nutrient agar, a loop full colony of bacteria was picked from a plate and put into 120ul of DNase/ RNase-free water in a sterile Eppendorf tube to obtain a turbid suspension of bacteria. The cell suspension was placed in an IWAKI THERMO ALUMI BATH at 95.0 ^{o}C for 10 minutes to lyse the cells and then placed on ice immediately. The Eppendorf tube containing the lysate was then centrifuged for 3 min at 10,000xg to pellet cell debris. The supernatant was then transferred to a new Eppendorf tube and used as a template DNA in polymerase chain reaction (PCR).

PCR and PCR Conditions for Pseudomonas aeruginosa

Universal bacterial 16s rRNA primers (Forward- 5’ AGAGTTTGATCCTGGCTCAG 3’, Reverse-5’ ACGGCTACCTTGTTACGACTT 3’) were used for PCR. DNA extracted from the bacteria suspension was used for PCR amplification. The PCR mixture consisted of 6.25 µl of

KOD Master Mix , 0.75µl of each primer (Reverse and Forward), 4.25ul of sterile distilled water and 1.0ul of bacterial DNA extract to make a final volume of 13µl. PCR amplification was performed using a Veriti200 Thermal cycler (Applied Biosystems) with an initial denaturation step of 98°C for 2.0 min, followed by 30 cycles at 98°C for 10 s , 52.0°C for 05s , annealing and a final extension step at 72°C for 2.0 min. The PCR products were visualized on 1.5% agarose gel electrophoresis following ethidium bromide staining.

3.14.3 PCR Amplicons Sequencing

Purifying the 16s PCR product.

The PCR product was transferred to a DNA miniprep spin column (Promega wizard® SV Gel and PCR clean-up Kit). Then 40.0ul of membrane binding solution was added to DNA miniprep spin column. The mixture was centrifuged at 8000xg for 1.0 minute and the filtrate was discarded. Then 500ul of membrane wash solution was added to DNA miniprep spin column, the column was centrifuged again at 8000xg for 1.0 minute, this step was repeated one more time; and each time the filtrate was discarded. Finally, 50.0ul of elution buffer was added to the DNA miniprep spin column. The column was allowed to stand for 1.0, then the column was placed in a 1.7-ml Eppendorf tube and centrifuged at 8000xg for 3.0 minutes. The purified DNA was collected in the Eppendorf tube.

Big dye PCR

Master mix for big dye was made as follows: Forward primer- big dye 0.5ul, Sequencing buffer 3.75ul forward primer 0.5ul, DNase/RNase free water 12.92ul and purified DNA template 2.0ul. Another master mix was made for the reverse primer as follows: big dye 0.5ul, Sequencing buffer 3.75ul reverse primer 0.5ul, DNase/RNase free water 12.92ul and purified DNA template

2.0ul. Then big dye PCR amplification was performed using a Veriti200 Thermal cycler (Applied Biosystems) with an initial denaturation step of 96°C for 1.0 min, followed by 25 cycles at 96°C for 10 s, 50.0°C for 5s, annealing and a final extension step at 60°C for 2.0 min. After big dye PCR cycles the DNA was precipitated.

Ethanol Precipitation

A master mix for ethanol precipitation was made as follows: 2.0ul of 3.0mM Sodium Acetate, 2.0ul of 125.0mM EDTA and 50.0ul of absolute ethanol to make a total of 54.0ul of master mix. 54.0ul of master mix was added to 20.0ul of big dye PCR product in a PCR tube and incubated in the dark at room temperature for 15.0 minutes. After incubation the mixture was centrifuged at 15000 rpm for 15.0 minutes. The supernatant was discarded. Then 200ul of 70% ethanol was added, the mixture was centrifuged again at 15000 rpm for 15.0 minutes, and the supernatant was discarded. The PCR tubes were then air dried at room temperature in the dark. After air drying, 20.0ul of formamide was added to the PCR tubes. The tubes were then placed in Veriti200 Thermal cycler (Applied Biosystems) for denaturation at 96°C for 2.0 min. after which the tubes were taken for sequencing.

Sequencing

After denaturation, the PCR tubes were transferred to SeqStudio™ Genetic Analyzer Applied biosystems by Thermo Fisher Scientific for sequencing according to manufacturer's instructions. After sequencing, the sequences were cleaned by editing-out non-standard DNA bases and aligned using Unipro UGENE version 45.0 software (Okonechnikov et al, 2012). The non-standard DNA bases were edited-out by comparing sequence chromatogram and the expected base in Unipro UGENE version 45.0 software. After alignment, contig sequence was generated.

The contig sequence was analyzed using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the bacteria species which was sequenced.

3.14.5 Antibiotic Resistance testing for Pseudomonas aeruginosa

Antibiotic resistance testing was conducted using VITEK 2S200 version 9.02 automated system (bioMérieux, Marcy l'Etoile, France) using AST-GN86 cards according to manufacturer's instructions. Antibiotic minimum inhibitory concentration (MIC) (Cefazolin, Ceftazidime, Cefepime, Imipenem, Gentamicin, Tobramycin, Ciprofloxacin, and Levofloxacin) data for the isolate was interpreted for susceptibility and resistance according to the VITEK2S200 version 9.02 Advanced Expert System and Clinical and Laboratory Standards Institute guidelines of 2017 (CLSI Ml00-S27).

Preliminary Establishment of *P. aeruginosa* infection

To establish *P. aeruginosa* infection dose, three 16-weeks old male hamsters were randomly selected and intranasally infected with 0.2 ml of 1.5 x 10^8 cfu/ml which translates to an effective dose of 3.0 x 10^7 cfu/ml *P. aeruginosa*, for a negative control, two randomly 16-week-old hamsters were intranasally administered with 0.2 ml of PBS pH 7.0. The hamsters were then sacrificed after 24 hours of being infected (Bhavsar et al., 2010; Coalson et al., 1986). General anaesthesia (xylazine 10mg/kg body weight Payton et al.,1993) was administered to the hamsters until no pedal withdrawal reflexes were present. The hamsters were sacrificed, and a nostril swab, trachea and lung tissue were collected. The lung and trachea were separately homogenised in 1.0 ml PBS pH 7.0. Then the homogenate was cultured in nutrient broth at 42^{o}C for 24 hours. After that a sub-culture was done on nutrient agar at 37^{o}C for 24 hours. Standard microbiology

biochemical tests for *P. aeruginosa* were conducted to identify the isolated organism. The hamsters used to establish infection dose were separate from the experimental groups but were from the same colonies as the experimental groups.

Infecting the Hamsters with *P. aeruginosa*

After 30 days of treatment, hamsters in group 1 to group 3 were intranasally inoculated with 0.2 ml of 1.5 x 10^8 cfu/ml which translates to an effective dose of 3.0 x 10^7 cfu/ml. After inoculating the hamsters with *P. aeruginosa,* the animals were put back in the cages for 18 hours and continued drinking water which contained drugs (Enalapril or Losartan) for the experimental animals and distilled water for the control animals. After 18 hours, temperature changes were measured every two hours for 36 hours and then once a day for three days using a Bo Hui infrared thermometer model T-168**.** Temperature was measured at the back of the neck with the thermometer touching the hamster**.** During the period of the experiment, the hamsters were checked to see if they had nasal discharge, lethargy, and changes in the appearance of fur. The hamsters were further observed for 14 days. The temperatures from each treatment were averaged to get one temperature reading. Average temperature = $\sum$ (temperature for hamsters in treatment group)/6. The average temperature was used to compare the infection outcomes.

3.15.0 Data management and analysis

The collected data was entered is Microsoft excel before being analyzed. The data was analyzed using IBM-SPSS version 27 software. Dunnett's multiple comparisons ANOVA (Dunnett's t-test) was used to compare the data between control hamsters and hamsters that were treated with enalapril or losartan. A p-value of $p < 0.05$ was considered significant for Dunnett's t-test. Further, a light microscope was used to analyze the tissues for inflammation and hyperplasia.

The histology slides were analyzed with the aid of three different histopathologists. To study effects of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) on the trachea barrier to *Pseudomonas aeruginosa* infection; Dunnett's Multiple Comparison ANOVA (Dunnett's t-test) was used to analyze the differences in temperature between experimental hamsters and control hamsters. A p value of $p < 0.05$ was considered significant for Dunnett's multiple comparisons, further the hamsters were checked for presence of nasal discharge, lethargy, and appearance of fur.

3.15.1 Interpretation of results

In this study, the concentrations of serum bradykinin, FOXA2, MUC5AC and MUC5B were compared in hamsters treated with enalapril or losartan to hamsters treated with distilled water (control). A p-value less than 0.05 was considered significant for Dunnett's t-test. Further, Dunnett's t-test was used to analyze the differences in temperature between experimental hamsters treated with enalapril or losartan and water (control), A p value less than 0.05 was considered significant for Dunnett's t-test. Furthermore, when studying the effects of ACEIs and ARBs on the tracheal mucosa barriers to *Pseudomonas aeruginosa* infection; fever was defined as temperature above 37.5^{o}C. If the experimental hamsters developed fever and control hamsters did not develop fever, it meant that enalapril and losartan had a negative effect on the respiratory immune system of hamsters since *P. aeruginosa* should not normally cause a disease in immune-competent hosts. For histopathological studies, the results were interpreted based on the criteria below:

Inflammatory reaction classification (Schafer et al., 2018 and Renne et al., 2009)

a) 0: None = Infiltration of mononuclear inflammatory cells (Lymphocytes, plasma cells, macrophages, and fibroblasts) less than 10% of the inflammation reaction

b) 1: Mild = Mononuclear inflammatory cells greater than 10% but less than 50% of inflammatory infiltrate

c) 2: Marked = Mononuclear inflammatory cells greater than 50% of inflammatory infiltrate

Hyperplasia classification: (Schafer et al., 2018 and Renne et al., 2009)

a. 0: None: = No hyperplasia

b. 1: Mild hyperplasia: = 2-3 cells thick,

c. 2: Moderate hyperplasia: = 4-6 cells thick,

d. 3: Marked hyperplasia: = Above 6 cells thick

CHAPTER FOUR: RESULTS

4.1 Effects of ACEIs and ARBs on concentration of serum bradykinin

Table 4.1.1 shows the minimum, maximum and mean concentration for Bradykinin. Table 4.1.1 shows that the mean concentration for hamsters treated with enalapril was highest at 33.21pg/mL followed by hamsters treated with losartan at 29.07 pg/mL and the last hamsters treated with water at 23.29 pg/mL. Figure 4.1.1 shows a bar graph of mean concentration of bradykinin in picograms per milliliter (pg/mL) and the error bars show that there were no significant differences ($p > 0.05$) in mean concentration between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval. Table 4.1.2 shows Dunnett's t-test of mean concentration of bradykinin and the table shows that there were no significant differences ($p > 0.05$) in the mean concentration of bradykinin between enalapril or losartan and water (control).

Table 4.1.1. shows the mean, minimum and maximum absorbance for bradykinin. Table 4.1.1 shows that the mean absorbance for enalapril was highest at 33.21 pg/mL followed by losartan at 29.07 pg/mL and the last water at 23.29 pg/mL.

Treatment	N	Mean (pg/mL)	Std. Deviation	Minimum (pg/mL)	Maximum (pg/mL)
Water	6	23.29	8.98	16.440	40.749
Enalapril	6	33.21	10.49	23.558	47.989
Losartan	6	29.07	17.85	15.066	62.119

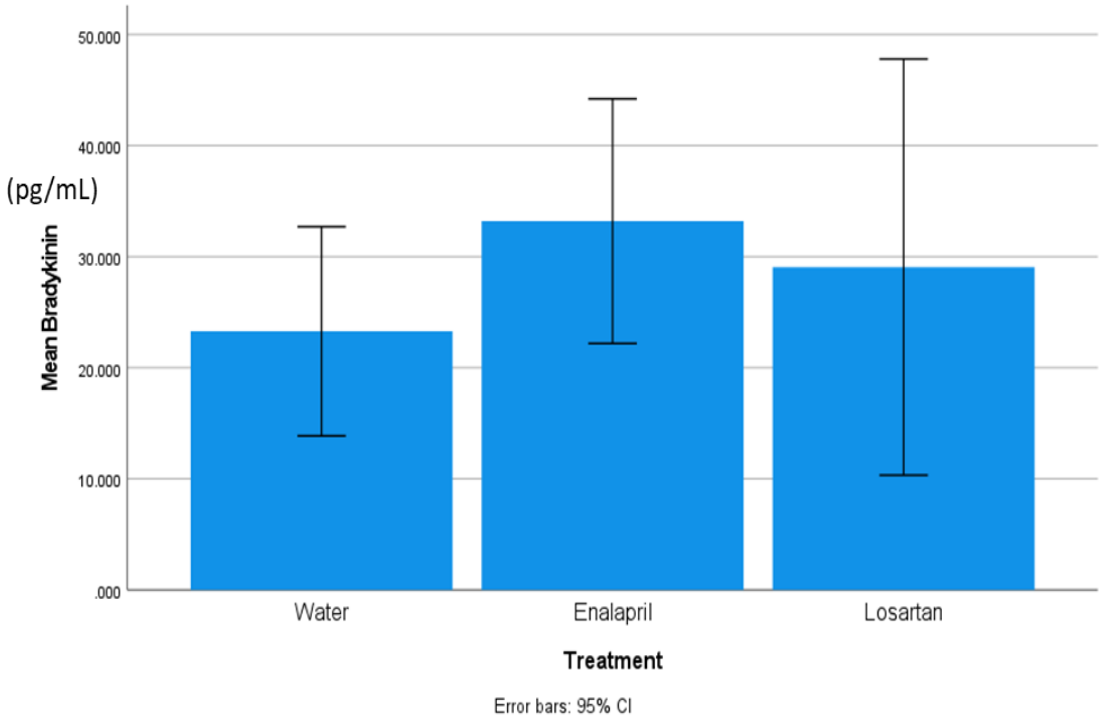

Figure 4.1.1 Shows mean concentration of bradykinin in picograms per milliliter (pg/mL) in hamsters treated with water, losartan, and Enalapril. The error bars on the bar graph shows that there were no significant differences in mean absorbance between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval.

Table 4.1.2 Dunnett's t-test. Table 4.1.2 shows that there were no significant differences ($p > 0.05$) in the mean bradykinin absorbances between enalapril or losartan and water (control).

Multiple Comparisons

Dependent Variable: Bradykinin

Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Treatment	(J) Treatment	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Enalapril	Water	9.92	7.52	0.340	-8.42	28.27
Losartan	Water	5.78	7.52	0.667	-12.57	24.13

a. Dunnett t-tests treat one group as a control and compare all other groups against it.

4.2 Effects of ACEIs and ARBs on MUC5AC, MUC5B and FOXA2

Table 4.2.1.0 shows the minimum, maximum and mean concentrations for FOXA2. Table 4.2.1.0 shows that the mean concentration of FOXA2 for hamsters treated with water was highest at 1095.14 pg/mL followed by Enalapril at 1065.39 pg/mL and the last hamster treated with Losartan at 1053.62 pg/mL. Figure 4.2.1.0 shows the bar graph for mean concentration of FOXA2 in picograms per milliliter (pg/mL) and the error bars show that there were no significant differences in mean concentration between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval. Table 4.2.1.1 shows Dunnett's t-test of mean concentration for FOXA2. Table 4.2.1.1 shows that there were no significant differences ($p > 0.05$) in the mean FOXA2 concentrations between enalapril or losartan and water (control).

Furthermore, Table 4.2.2.0 shows the minimum, maximum, and mean concentration for MUC5AC. Table 4.2.2.0 shows that the mean concentration for hamsters treated with Enalapril was the highest 629.68 pg/mL followed by Losartan at 599.48 pg/mL and hamsters treated with water was the last at 476.07 pg/mL. Figure 4.2.2.0 shows the bar graph of mean concentration of MUC5AC picograms per milliliter (pg/mL) and the bar graph shows that there was no significant difference in mean concentration between hamsters treated with Enalapril or Losartan and hamsters treated with water (control) at 95% confidence interval. Table 4.2.2.1 shows Dunnett's t-test of mean concentration for MUC5AC. Table 4.2.2.1 shows that there were no significant differences ($p > 0.05$) in the mean concentration of MUC5AC between hamsters treated with enalapril or losartan and hamsters treated with water (control).

Similarly, Table 4.2.3.0 shows the minimum, maximum, and mean concentration for MUC5B.Table 4.2.3.0 shows that Losartan had the highest mean concentration at 619.98 pg/mL followed by Enalapril 604.42 pg/mL and the last water at 515.74 pg/mL. Figure 4.2.3.0 shows the bar graph of MUC5B mean concentration in picograms per milliliter (pg/mL). The bar graph shows that there were no significant differences in the mean concentration of MUC5B between hamsters treated with Enalapril or Losartan and hamsters treated with water (control) at 95% confidence interval. Table 4.2.3.1 shows Dunnett's t-test of mean concentration of MUC5B. Table 4.2.3.1 shows that there were no significant differences ($p > 0.05$) in the mean concentration of MUC5B between enalapril or losartan treated hamsters and water treated hamsters (control).

Table 4.2.1.0 Shows mean, standard deviation, minimum, and maximum concentration for FOXA2 in hamsters treated with water, losartan, and enalapril. Table 4.2.1.0 shows that the mean absorbance of water was highest in control at 1095.14 pg/mL followed by enalapril at 1065.39 pg/ml and the last losartan at 1053.62 pg/mL.

Treatment	N	Mean (pg/mL)	Std. Deviation	Minimum (pg/mL)	Maximum (pg/mL)
Water	6	1095.14	69.53	976.21	1158.71
Enalapril	6	1065.39	40.04	1000.32	1121.20
Losartan	6	1053.62	42.11	994.89	1103.37

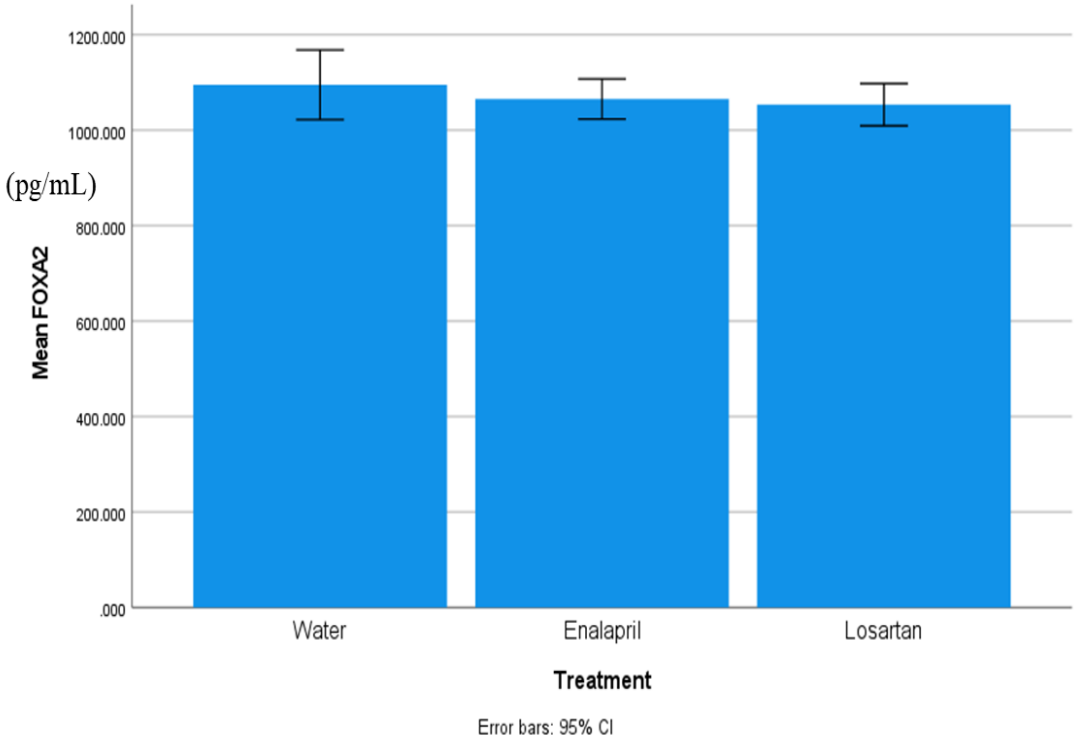

Figure 4.2.1.0 Shows mean absorbances for FOXA2 in hamsters treated with water, losartan, and Enalapril. The error bars on the bar graph show that there were no significant differences in mean concentration of FOXA2 between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval.

Table 4.2.1.1 Shows FOXA2 Dunnett's t-test. Table 4.2.1.1 shows that there is no significant difference in FOXA2 concentration ($p > 0.05$) when comparing hamsters that were treated with enalapril or losartan, and hamsters that were treated with water.

Multiple Comparisons

Dependent Variable: FOXA2

Dunnett t (2-sided)[a]

		Mean			95% Confidence Interval	
(I) Treatment	(J) Treatment	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Enalapril	Water	-29.74	30.20	0.527	-103.42	43.93
Losartan	Water	-41.52	30.20	0.313	-115.19	32.15

a. Dunnett t-tests treat one group as a control and compare all other groups against it.

Table 4.2.2.0 Shows the mean, standard deviation, minimum, and maximum absorbances of MUC5AC in hamsters treated with water, losartan, and enalapril. Table 4.2.2.0 shows that the mean concentration for enalapril was the highest 629.68 pg/mL followed by losartan at 599.48 pg/mL and water at 476.07 pg/mL.

Treatment	N	Mean (pg/mL)	Std. Deviation	Minimum (pg/mL)	Maximum (pg/mL)
Water	6	476.07	190.94	353.48	846.28
Enalapril	6	629.68	87.46	487.81	744.55
Losartan	6	599.48	84.15	511.08	752.74

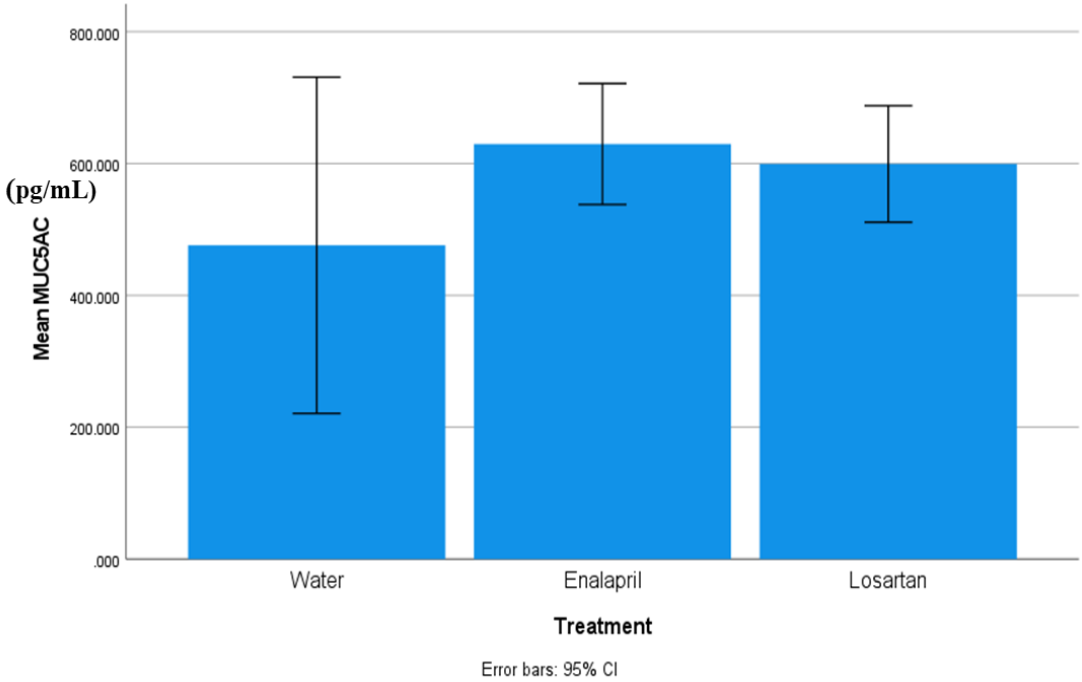

Figure 4.2.2.0 Shows mean absorbances for MUC5AC in hamsters treated with water, losartan, and Enalapril. The error bars on the bar graph show that there were no significant differences between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval.

Table 4.2.2.1 Shows Dunnett's t-test. Table 4.2.2.1 shows that there are no significant differences in MUC5AC concentration ($p > 0.05$) when comparing hamsters treated with enalapril or losartan, and water.

Multiple Comparisons

Dependent Variable: MUC5AC

Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Treatment	(J) Treatment	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Enalapril	Water	153.62	75.42	0.105	-30.34	337.57
Losartan	Water	123.41	75.42	0.209	-60.55	307.37

a. Dunnett t-tests treat one group as a control and compare all other groups against it.

Table 4.2.3.0 Shows the mean, standard deviation, minimum, and maximum absorbances for MUC5B in hamsters treated with water, losartan, and enalapril. Table 4.2.3.0 shows that Losartan had the highest mean absorbance at 619.98 pg/mL followed by enalapril 604.42 pg/mL and the last water at water at 515.74 pg/mL.

Treatment	N	Mean(pg/mL)	Std. Deviation	Minimum(pg/mL)	Maximum(pg/mL)
Water	6	515.74	140.55	354.48	743.76
Enalapril	6	604.42	61.21	523.23	693.55
Losartan	6	619.98	69.69	495.92	683.59

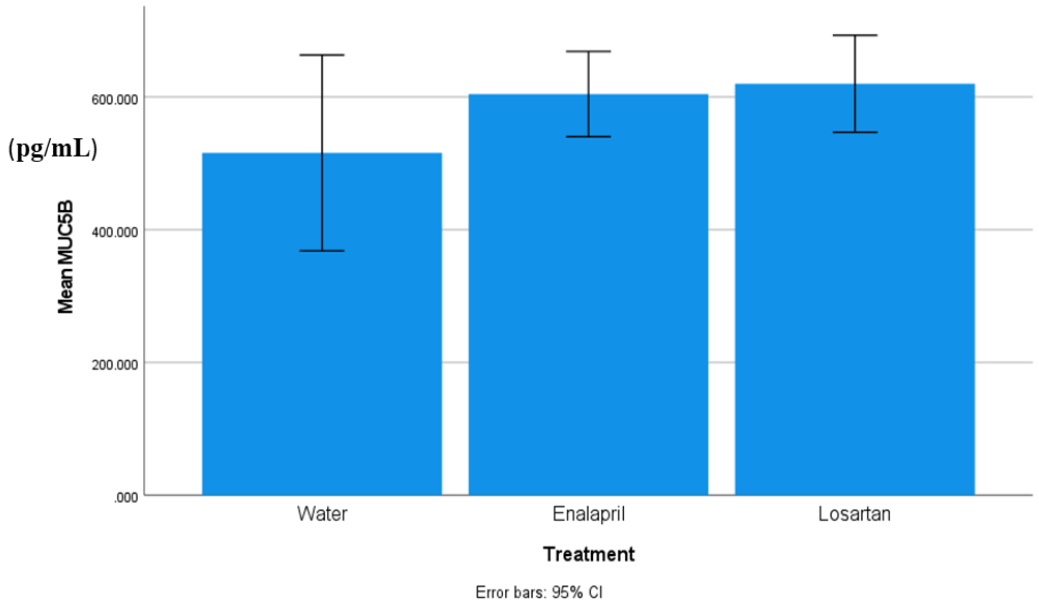

Figure 4.2.3.0 Shows mean concentration for MUC5B in picograms per milliliter (pg/mL) in hamsters treated with water, losartan, and Enalapril. The error bars on the bar graph show that there were no significant differences in mean concentration between hamsters treated with Enalapril or Losartan and hamsters treated with water (Control) at 95% confidence interval.

Table 4.2.3.1 Shows MUC5B multiple comparisons ANOVA. Table 4.2.3.1 shows that there is no significant difference in the concentration of MUC5AC ($p>0.05$) when comparing hamsters that were treated with enalapril or losartan, and water.

Multiple Comparisons

Dependent Variable: MUC5B

Dunnett t (2-sided)[a]

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Enalapril	Water	88.68	56.13	0.229	-48.23	225.60
Losartan	Water	104.24	56.13	0.145	-32.68	241.16

a. Dunnett t-tests treat one group as a control and compare all other groups against it.

4.3 Effects of ACEIs and ARBs on Histology of Tracheal Mucosa

Figures 4.3.1.0 to 4.3.1.5 show histological sections of the hamster trachea that were treated with enalapril. Figures 4.3.1.0 to 4.3.1.3 show mild inflammatory reactions; Two of a total of six hamsters that were treated with enalapril had a mild inflammatory reaction, while none of the hamsters treated with losartan or water showed signs of inflammation. Figure 4.3.1.3 shows submucosal glands and figures 4.3.1.4 to 4.3.1.5 show the epithelia of a hamster that were treated with enalapril; there was no hyperplasia of the goblet cells or submucosal glands that was observed. Figures 4.3.2.0 to Figure 4.3.2.2 show histological sections of a hamster that was treated with losartan. Figure 4.3.2.0 shows a section of the submucosal glands, while Figures 4.3.2.1 and 4.3.2.2 show epithelia of the trachea. In both the submucosal glands and the goblet cells, hyperplasia was not observed. Figures 4.3.3.0 to Figure 4.3.3.2 show histological sections of a hamster that was treated with distilled water (negative control). Figure 4.3.3.0 shows a section of the submucosal glands, while Figures 4.3.3.1 and 4.3.3.2 show the epithelia. In both the submucosal glands and the goblet cells, no hyperplasia was seen.

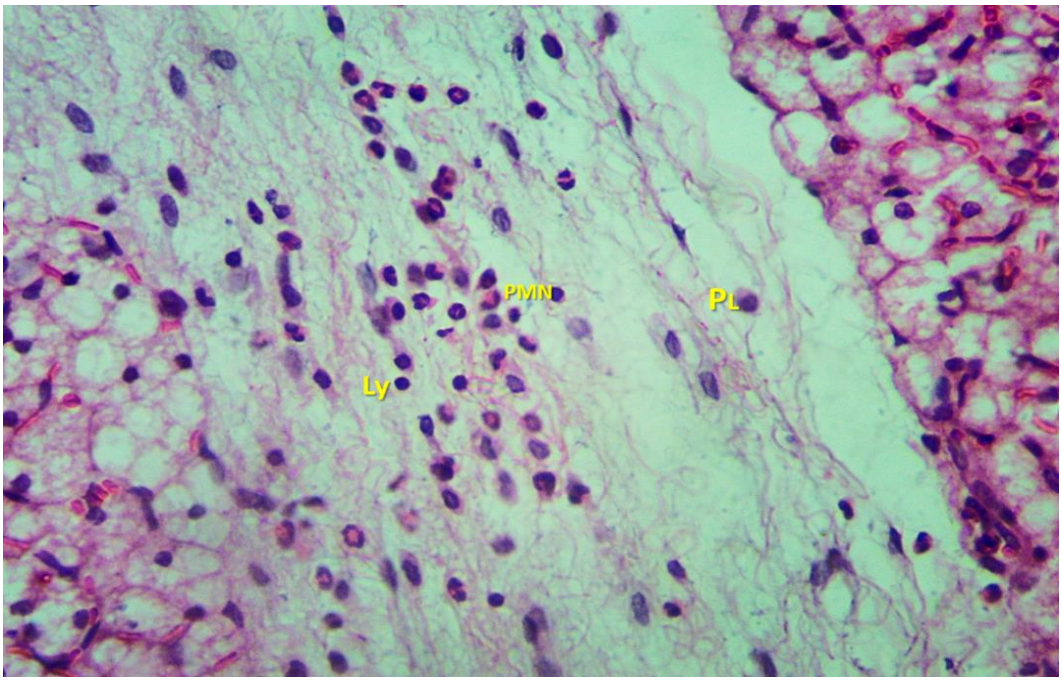

Figure 4.3.1.0 H&E 400X magnification section of the trachea of a hamster that was treated with enalapril showing mild inflammation. PMN= Polymorphonuclear cells (Neutrophils, eosinophils, mast cells and basophils) Ly = lymphocyte: PL= plasma cell.

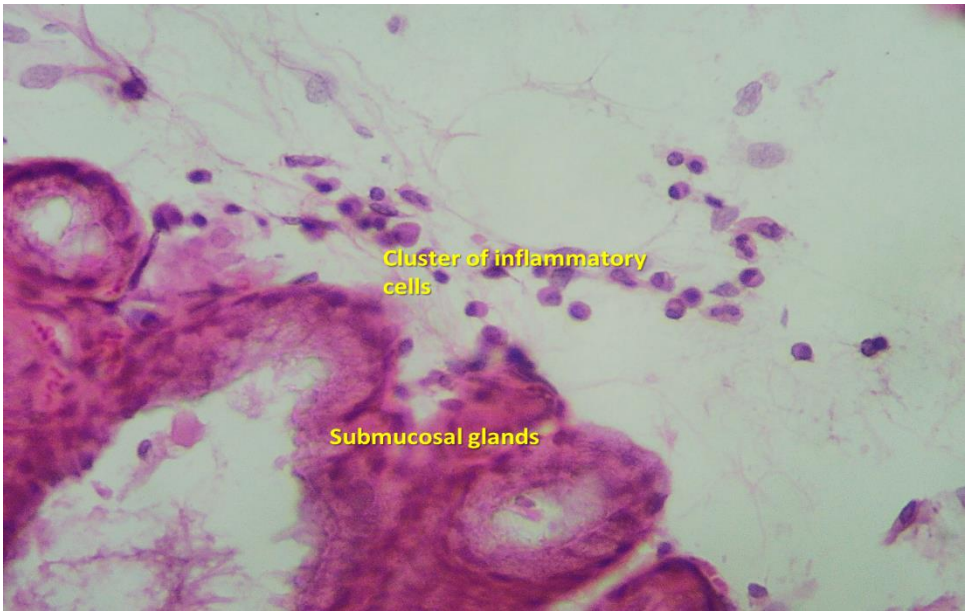

Figure 4.3.1.1 H&E 400X magnification Section of the trachea of a hamster that was treated with enalapril showing a cluster of plasma cells near the submucosal glands.

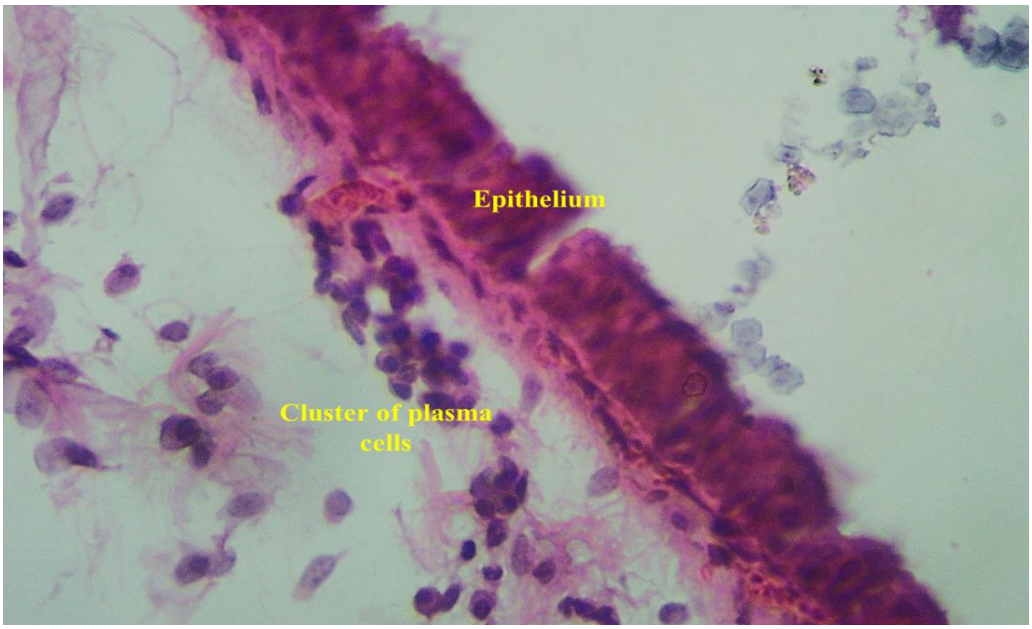

Figure 4.3.1.2 H&E 400X magnification Section of the trachea for a hamster that was treated with enalapril showing a cluster of plasma cell near the below the epithelium

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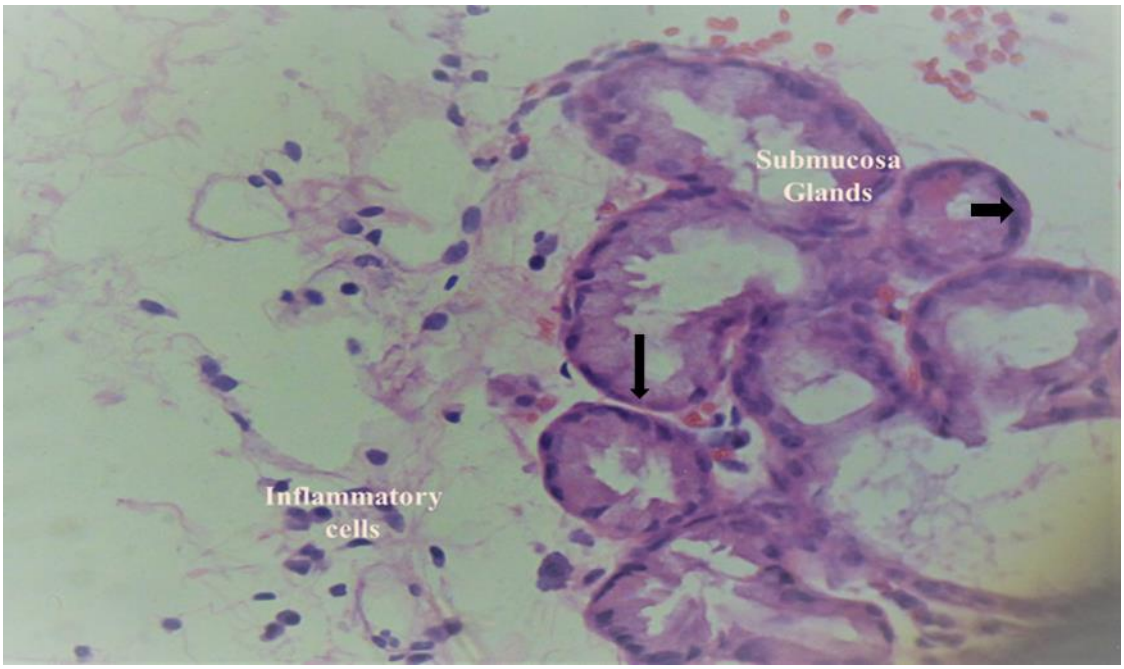

Figure 4.3.1.3 H&E 400X magnification section of the trachea for a hamster that was treated with enalapril showing submucosal glands with one layer of cells (black arrows), and inflamtory cells. The glands have a regular shape and no hyperplasia was observed.

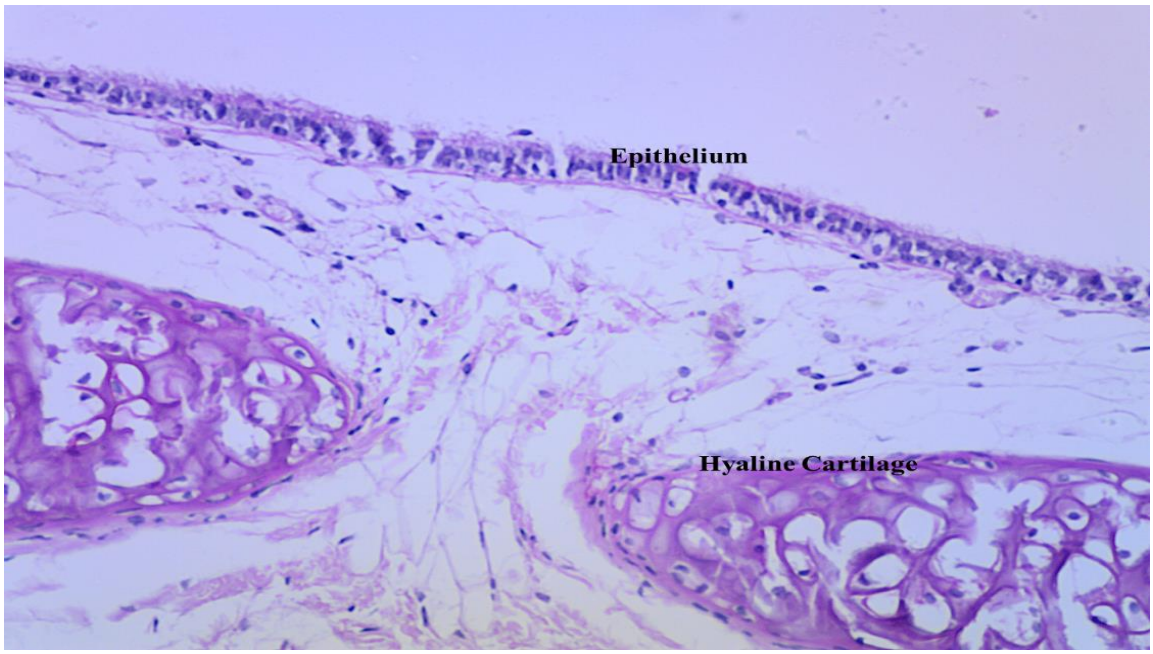

Figure 4.3.1.4 PAS 100X magnification section of the trachea for a hamster that was treated with enalapril showing the epithelium. In this section, hyperplasia of goblet cells was not seen.

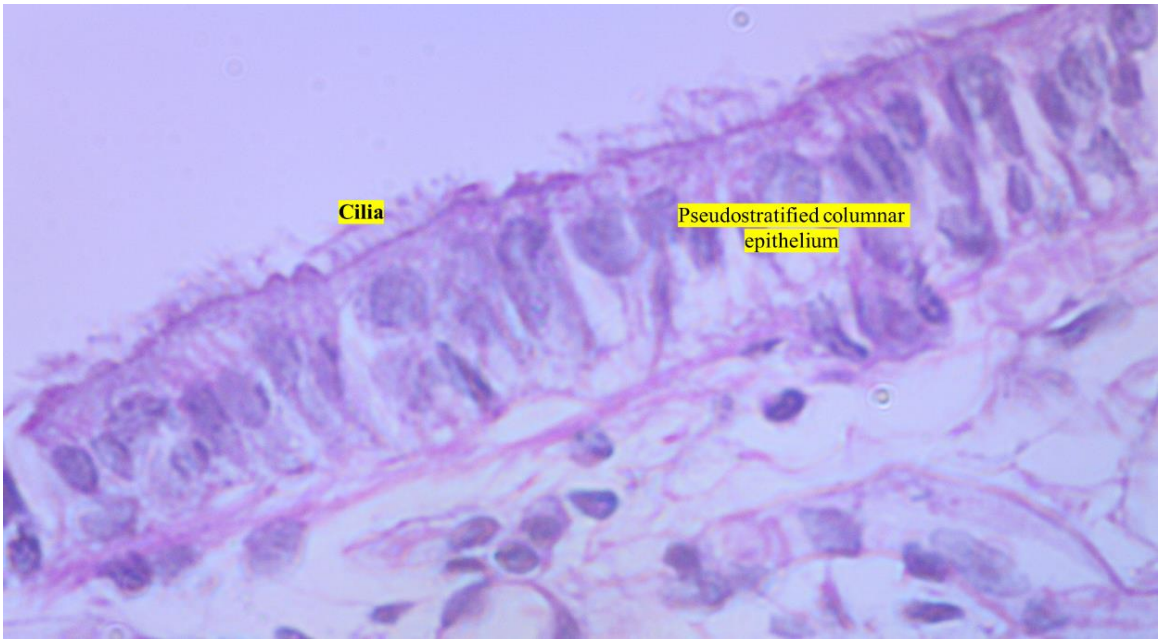

Figure 4.3.1.5 PAS 400X magnification section of the trachea for a hamster that was treated with enalapril showing the epithelium. Hyperplasia of goblet cells was not observed.

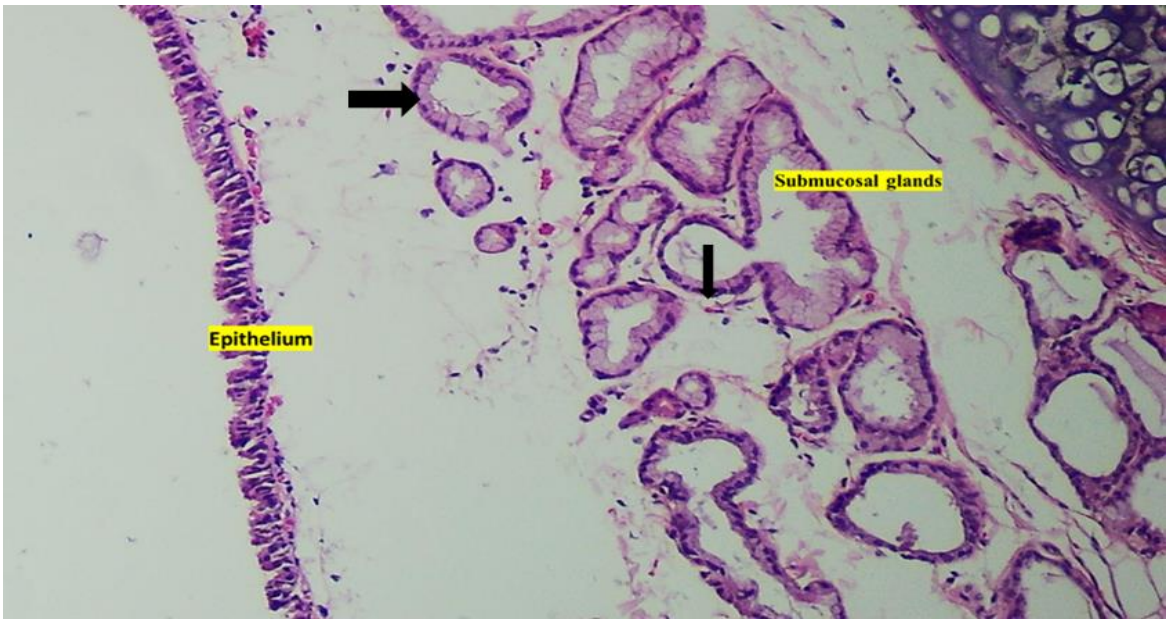

Figure 4.3.2.0 H&E 100X magnification section of the trachea of a hamster treated with Losartan showing submucosal glands with one layer of cells (black arrows). Hyperplasia of the glands was not seen.

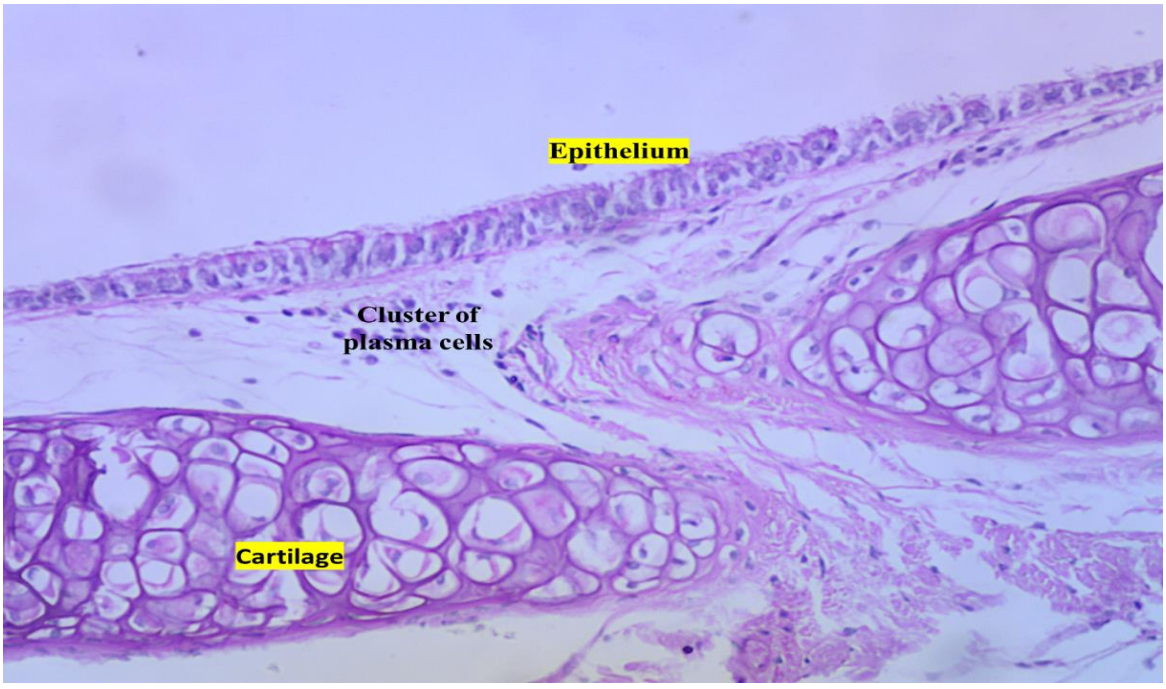

Figure 4.3.2.1 PAS 100X section of the trachea for a hamster that was treated with Losartan showing submucosal glands with one layer of cells.Hyperplasia of the of goblet cells was not seen

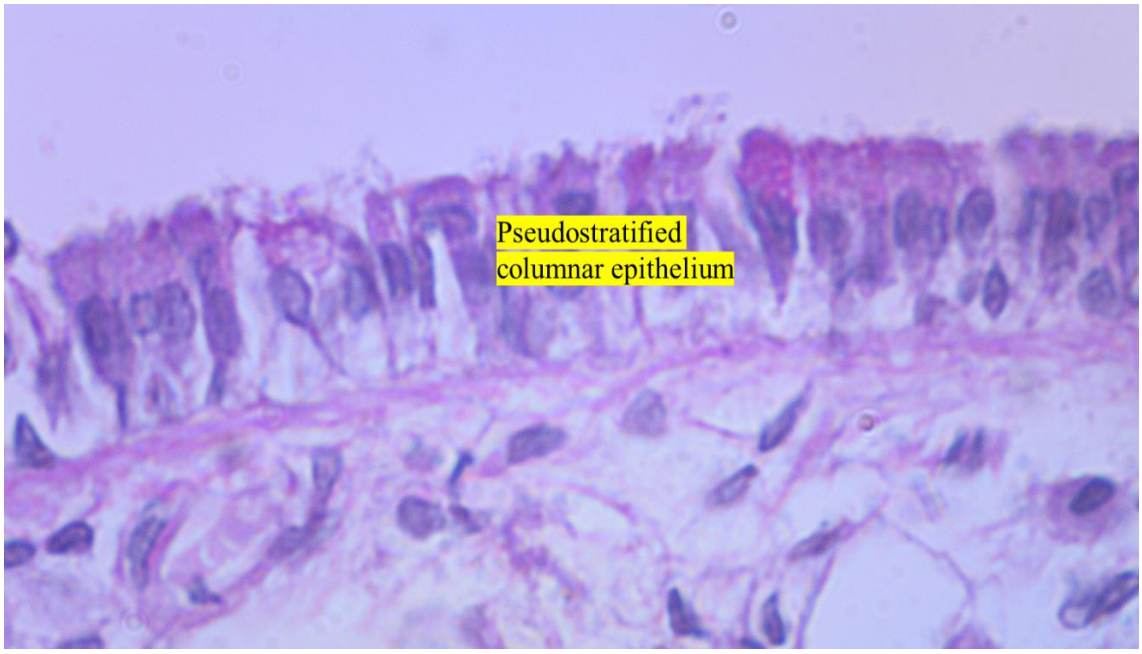

Figure 4.4.2.2 PAS 400X section of the trachea of a hamster that was treated with Losartan and showed respiratory epithelium. No hyperplasia of the goblet cells was seen.

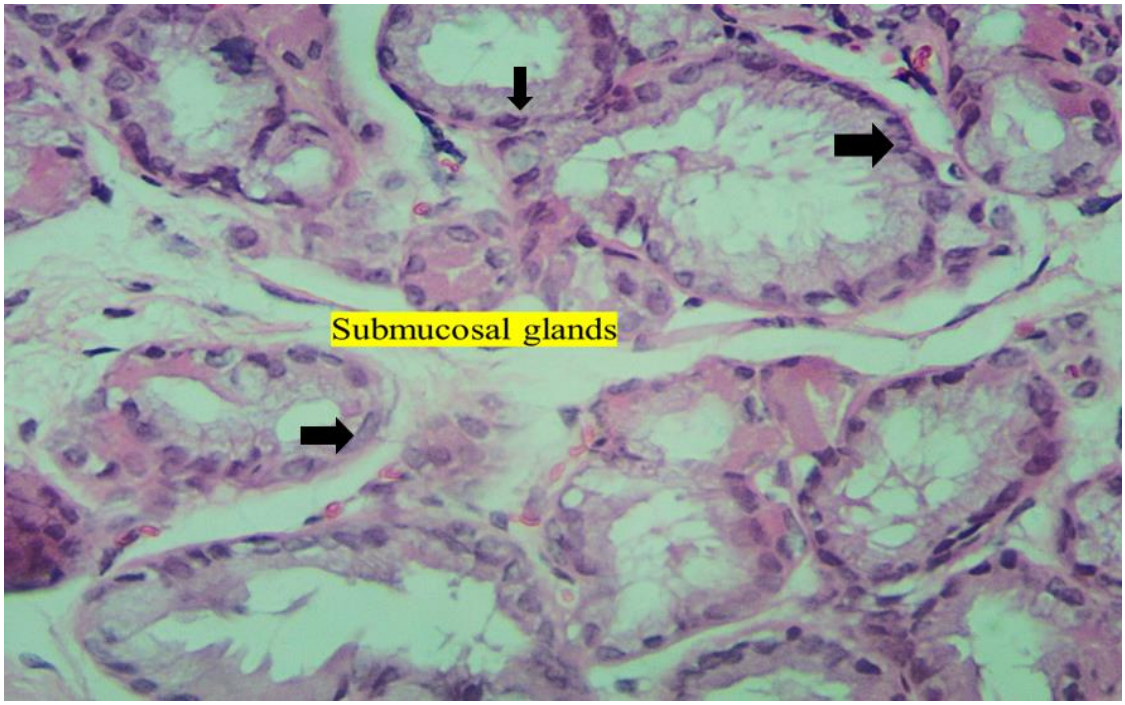

Figure 4.3.3.0 H&E 400X magnification section of the trachea of a hamster that was treated with water (control) showing submucosal glands with one layer of cells (Black arrows). No hyperplasia of the glands was seen.

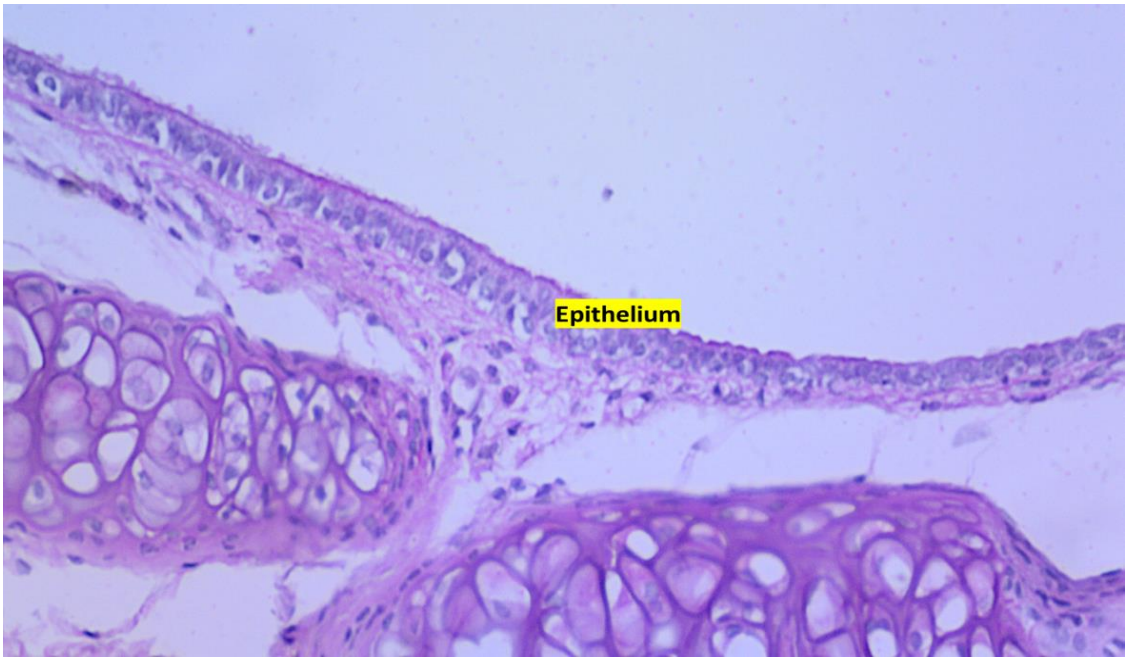

Figure 4.3.3.1 PAS 100X section of the trachea of a hamster treated with water (control) showing respiratory epithelium.Hyperplasia of the goblet cells was not seen.

Figure 4.3.3.2 PAS 400X section of the trachea for a hamster treated with water (control) showing respiratory epithelium. Hyperplasia of the goblet cells was not seen.

4.4 Effects of ACEIs and ARBs on the tracheal mucosa barriers to *Pseudomonas aeruginosa* infection

Figure 4.4.1 shows the growth of the isolate collected from a patient on nutrient agar at 37°C after 24 hours. Figure 4.4.2 shows ethidium bromide staining for 16s PCR amplicons and Table 4.4.1 shows the contig sequences of the isolate. Table 4.4.2 shows antibiotic resistance profile for *Pseudomonas aeruginosa* PA1. Table 4.4.3 shows the results for establishing infection dose for *P. aeruginosa.* The table shows that *P. aeruginosa* was isolated from the nostril, trachea, and lungs of the hamsters that were infected with the isolate, while *P. aeruginosa* was not isolated from negative controls. During the experiment, the mean, minimum, and maximum temperatures of the hamsters from group 1 to group 4 were recorded as shown in Table 4.4.4. The temperature in all treatment groups ranged from 35.53 ℃ to 37.2 ℃ with an average of 36.06 ℃. The hamsters were further observed for 14 days to see if any mortality would be recorded; no mortality was recorded after 14 days from all treatment groups (Group 1 to group 4). Additionally, no nasal discharge or lethargy were observed, and the appearance of fur was comparable to the hamsters that were administered with PBS. Figure 4.4.3 shows a bar graph of mean temperature with error bars. The bar graph shows that there were no significant differences in mean temperatures between hamsters in group 1 to group 3 compared to the hamsters in group 4 at 95% confidence interval. Table 4.4.5 shows Dunnett's t-test of mean temperature among group 1 to group 3 and group 4. Table 4.4.5 shows that there was no significant difference ($p > 0.05$) in mean temperature between hamsters in group 1 to group 3 and hamsters in group 4.

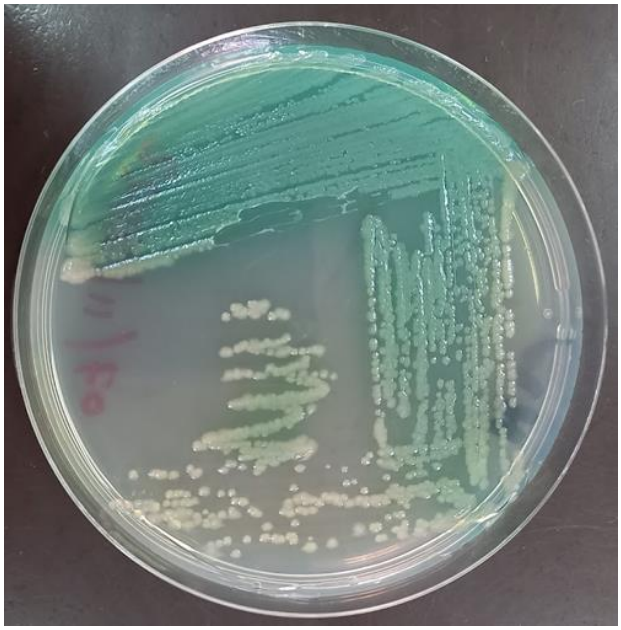

Figure 4.4.1. Shows Nutrient agar growth of P. aeruginosa after 24 hours Nutrient agar culture.

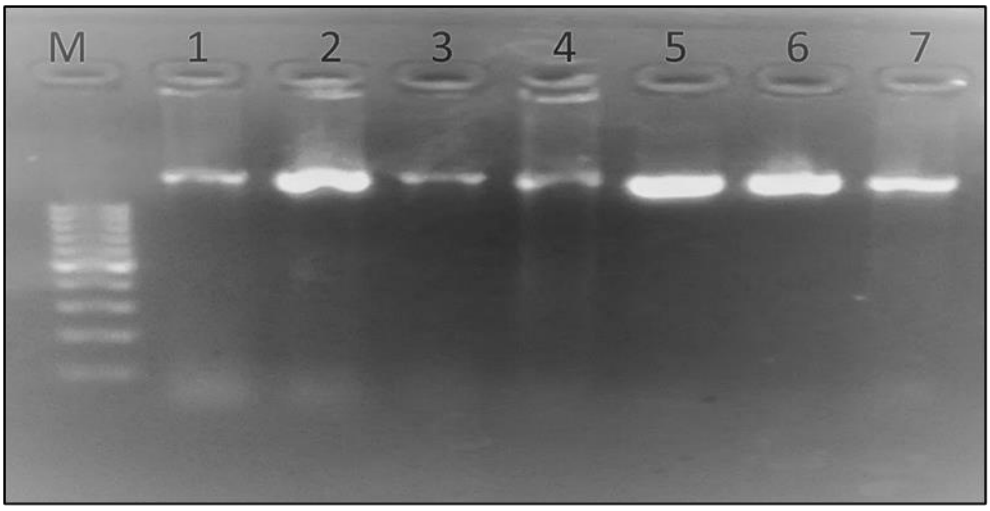

Figure 4.4.2 Shows ethidium bromide staining of the 16s PCR product. The amplicon of interest is labelled number 5. M = Ladder marker, Numbers 1 to 7 = 16s PCR amplicons

Table 4.4.1 Shows Contig sequence for *Pseudomonas aeruginosa* that was blast in NCBI database. The organism was identified as Pseudomonas aeruginosa PA1. Accession number: MK685346.1

>Contig

TAGGCCATGGCGACCTATATATACCGTGCCTACTTGCGGTTAGACTAGCTACTTCTG
GAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTG
CAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTG
GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATG
ATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTG
CCCACCCGAG

Table 4.4.2 Shows antibiotic resistance results for *Pseudomonas aeruginosa* PA1.

Selected Organism	***Pseudomonas aeruginosa* PA1**				
Antimicrobial	MIC	Interpretation	ANTIMICROBIAL	MIC	Interpretation
Cefazolin	≥ 64	R	Imipenem	2	S
Ceftazidime	16	I	Gentamicin	≥16	R
Cefepime	32	R	Tobramycin	4	S
			Ciprofloxacin	≤ 0.25	S
			Levofloxacin	1	S

Key: S=sensitive R= Resistant I= Intermediate and MIC=minimum inhibitory concentration.

Table 4.4 3 Establishing *P. aeruginosa* infection dose in Hamsters.

Treatment	**Nostril**	**Trachea**	**Lung**
0.2ml PA	Positive	Positive	Positive
0.2ml PA	Positive	Positive	Positive
0.2ml PA	Positive	Positive	Positive
0.2ml PBS	Negative	Negative	Negative
0.2ml PBS	Negative	Negative	Negative

Key 0.2ml PA = 0.2ml of 1.5 x 10^8 cfu/ml of Pseudomonas aeruginosa. Positive= P. aeruginosa was isolated. Negative = P. aeruginosa was not isolated

Table 4.4.4 Shows Minimum, Maximum and Mean temperature for the hamsters.

Treatment	N	Mean (°C)	Std. Deviation	Minimum (°C)	Maximum (°C)
Group 1	18	36.04	0.36	35.53	37.23
Group 2	18	36.02	0.17	35.67	36.30
Group 3	18	36.11	0.21	35.83	36.53
Group 4	18	36.07	0.21	35.60	36.40
Total	72	36.06	0.24	35.53	37.23

Key: *N= number of times temperature was taken.* Group 1= (distilled water+ *P. aeruginosa*), Group 2= (Enalapril + *P. aeruginosa*), Group 3= (Losartan+ *P. aeruginosa*), and Group 4 = (distilled water + PBS)

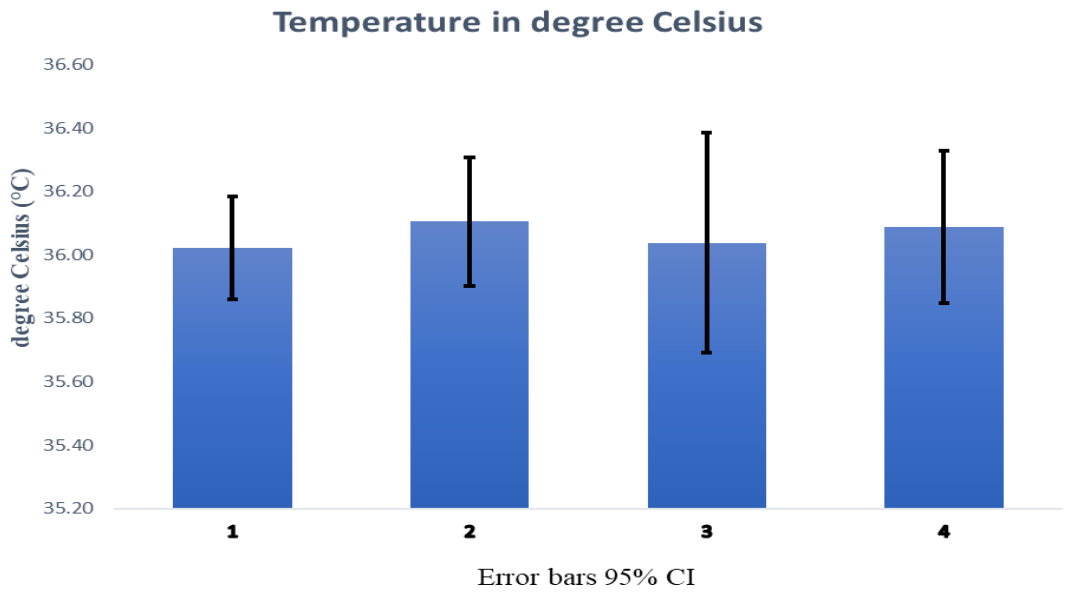

Figure 4.4.3 Shows a bar graph with error bars of mean temperature changes in hamsters from group 1 to group 4. The bar graph shows that there is no significant difference between mean temperature of hamsters in group 4 and the mean temperature of hamsters in group 1 to group 3 at 95% confidence interval. In the bar graph 1=group 2 (enalapril+ *P. aeruginosa*), 2= group 3 (losartan + *P. aeruginosa*), 3= group 1 (distilled water + *P. aeruginosa*) and 4= group 4 (distilled water + PBS)

Table 4.4.5 shows Dunnett's t-test. The table shows that there is no significant difference ($p > 0.05$) in temperature between hamsters treated with enalapril or losartan and the control.

Multiple Comparisons

Dependent Variable: Temperature

Dunnett t (2-sided) [a]

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Group 1	Group 4	-0.02	0.08	0.97	-0.23	0.17
Group 2	Group 4	-0.04	0.08	0.91	-0.24	0.15
Group 3	Group 4	0.03	0.08	0.94	-0.16	0.24

a. Dunnett t-tests treat one group as a control and compare all other groups against it.

Key: Group 1= (distilled water+ *P. aeruginosa*), Group 2= (Enalapril + *P. aeruginosa*), Group 3= (Losartan+ *P. aeruginosa*), and Group 4 = (distilled water + PBS)

CHAPTER FIVE: DISCUSSION

This study aimed to investigate the effects of angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEIs) on the tracheal mucosa barriers to *Pseudomonas aeruginosa* infection in order to understand the effects of these drugs on human patients taking these drugs to manage hypertension and other cardiovascular related diseases. Although ARBs and ACEIs are associated with a dose dependent induction of cough and angioedema as their side effects, no literature was found that documents the effects of ARBs and ACEIs on FOXA2, MUC5B and MUC5AC. FOXA2, MUC5B and MUC5AC are important macromolecules in maintenance of respiratory tract homeostasis and mucociliary escalator (Bonser & Erle 2017; Choi et al., 2020). The tracheal epithelial barrier is made up of three components, one of which is the mucociliary escalator (Ganesan et al., 2013). Impairment of the mucociliary escalator can lead to increased susceptibility of the respiratory system to infections. Mucociliary escalator is the primary innate defense mechanism of the respiratory tract (Bustamante-Marin & Ostrowski, 2017). Mucociliary escalator can be impaired if there is an over expression of respiratory mucins specifically, MUC5AC and MUC5B (Singanayagam et al.,2022; Bonser and Erle, 2017).

5.1 Effects of Angiotensin Receptor Blockers (ARBs) And Angiotensin Converting Enzyme Inhibitors (ACEIs) On serum Bradykinin in a Syrian Hamster.

ACEIs increase bradykinin by reducing the activity of angiotensin converting enzyme (ACE) (Karamyan, 2021, Campbell et al., 2005). Bradykinin degradation is done by angiotensin converting enzyme (Tabassum et al., 2022), therefore, inhibiting ACE inadvertently inhibits bradykinin degradation (Schmaier, 2019). Further, ARBs increase levels of bradykinin by reducing the activity of neutral endopeptidase (Walther et al., 2002); neutral endopeptidase are important in the metabolism of bradykinin (Nowak et al., 2011). Bradykinin is a potent, transient vasoactive peptide that acts as a vasodilator and an inflammatory mediator in various signaling cascades (Rex et al., 2022). Bradykinin contracts airway smooth muscles, increases microvascular leakage, and stimulates mucus secretion (Ellis & Fozard, 2002; Barnes,1992). Increased secretion of mucus in the trachea can negatively affect mucociliary clearance. The findings of this study show that hamsters that were treated with Enalapril (an ACEI) or Losartan

(an ARB) had increased amount of serum bradykinin compared to the control hamsters (Table 4.1.1), however, the increase in bradykinin was not significant ($p > 0.05$) (Table 4.1.2; Figure 4.1.1). Some studies have shown that ACEIs increase bradykinin levels significantly. For example, a study by Burger et al. (2014) found that lisinopril significantly increased plasma bradykinin in mice. Other studies have however found that ACEIs and ARBs do not significantly increase bradykinin levels. A study by Stanziola et al. (1999), found that chronic use of ACEIs induced alternative mechanisms for bradykinin metabolism. Additionally, ARBs do not inhibit ACE which is the most important enzyme in bradykinin metabolism (Smolinska et al., 2023; Blais et al., 1997). This could explain why the increase in bradykinin in this study was not significant. Since, bradykinin levels where not significantly increased, the implication is that taking losartan or enalapril may not cause an increase in mucus secretion of the respiratory tract.

5.2 Effects of ARBs and ACEIs on MUC5AC, MUC5B and FOXA2 of a hamster

Abnormalities in mucus secretion, including increased expression of the main airway mucins MUC5AC and MUC5B, are an important characteristic of inflammatory airway diseases (Singanayagam et al., 2022). Forkhead box protein A2 (FOXA2) is a key transcriptional regulator that maintains airway mucus homeostasis, depletion of FOXA2 leads to mucus hypersecretion which leads to worsening of respiratory system infections (Choi et al., 2020; Wan et al., 2004). The results of this study show that enalapril and losartan decreased the concentration of FOXA2 compared to the control (table 4.21.0), however, the decrease in concentration of FOXA2 was not significant ($p > 0.05$) compared to the control (table 4.2.1.1). Based on these results, enalapril and losartan did not lead to a significant reduction in FOXA2 in the trachea. No literature was found the shows the effect of ARBs and ACEIs on respiratory FOXA2. Since there was significant reduction in FOXA2, taking of enalapril or losartan would not lead to dysregulation of mucus homeostasis.

In chronic inflammatory respiratory disorders, mucine-5B (MUC5B) and mucin-5AC (MUC5AC) increase in the respiratory tract (Silva and Bercik, 2012), a higher ratio of MUC5AC to MUC5B is correlated with type-2 inflammation (Lachowicz-Scroggins et al., 2016). MUC5B and MUC5AC are the main matrix-forming macromolecules in airway mucus, because of this,

viscoelastic properties of airway mucus depend on them (Bonser and Erle, 2017). Thick mucus slows the mucociliary escalator, affecting the ability of the respiratory track to remove pathogens and other particles. Loss of MUC5B inhibits innate inflammatory responses, resulting in accumulation of alveolar macrophages with impaired ability to phagocytose and clear pathogens (Huang et al., 2022). Furthermore, MUC5AC confers resistance to viral infection (Ehre et al., 2005), however, overexpression of MUC5AC is detrimental in acute lung injury as it may cause mucus plugs and increase bronchial hyperresponsiveness (Koeppen et al., 2013, Evans et al., 2015). MUC5AC-rich mucus markedly impairs mucociliary transport because it is tethered to the epithelial wall (Bonser et al., 2016). Mucins selectively bind to and disrupt the aggregation of microbial pathogens, preventing pathogens from entering the periciliary layer and blocking their access to the surfaces of the underlying epithelial cell surfaces (Whitsett, & Alenghat 2015). The findings of this study show that Enalapril and Losartan both caused an increase in MUC5AC and MUC5B (table 4.2.2.0). However, the increase was not significant ($p > 0.05$) compared to the control (table 4.2.3.0). Based on these results, Enalapril, and Losartan increases MUC5AC and MUC5B, but the increase is not significant ($p > 0.05$) when compared to the control. No other studies were found that show the effects of ARBs and ACEIs on MUC5AC and MUC5B. Further studies are required to study what effect these marginal increases may have on the viscoelasticity properties of respiratory mucus and mucociliary escalator (Bustamante-Marin & Ostrowski, 2017). Angiotensin Receptor Blockers and Angiotensin Converting Enzyme Inhibitors have no significant effect on respiratory FOXA2, MUC5AC and MUC5B of the Hamster tracheal Mucosa. Therefore, taking these drugs would not negatively affect the mucociliary escalator by overexpression of respiratory mucins.

5.3 Effect of ARBS and ACEIS on the hamster tracheal goblet cells and submucosal glands.

The main objective of this study was to find out if ARBs and ACEIs can cause hyperplasia of the goblet cell, and Submucosal gland. Hyperplasia of goblet glands and submucosal glands can cause an increase in mucus production and impair the mucociliary escalator, which can lead to worsening of respiratory infections (Singanayagam et al.,2022; Bonser et al., 2016; Rose & Voynow, 2006). From the histopathological sections, this study shows that, enalapril and losartan did not cause hyperplasia of the goblet cells or submucosal glands. The hamsters in all treatment groups did not develop goblet cell hyperplasia or submucosal gland hyperplasia. Airway goblet

cell hyperplasia (GCH) and macrophage infiltrate are pathological features present in many chronic respiratory disorders. Macrophages induce MUC5B expression in bronchiolar goblet cell hyperplasia, suggesting a role for them in the pathogenesis of airway MUC5B-related GCH (Silva and Bercik, 2012). Similarly, the Forkhead box protein A2 (FOXA2) is a key transcriptional regulator that maintains airway mucus homeostasis (Choi et al., 2020; Wan et al., 2004). Depletion of FOXA2 results in goblet cell hyperplasia, which leads to mucus hypersecretion (Choi et al., 2020; Choi, Choe, and Lau, 2020). Inflammatory conditions such as angioedema may deplete FOXA2 expression and lead to hyperplasia of goblet cells and submucosal glands (Choi, Choe & Lau., 2020). This study also found that ARBs and ACEIs did not significantly reduce the concentration of FOXA2 in the trachea, this could explain why goblet cell hyperplasia or submucosal gland hyperplasia was not observed (Choi et al 2019; Choe and Lau, 2020). Overall, the results suggest that taking enalapril or losartan cannot lead to overexpression of mucus in the respiratory tract via hyperplasia of mucin producing glands.

5.4 Effects of ACEIs and ARBs on mucosal barriers to *Pseudomonas aeruginosa* infection

Pseudomonas aeruginosa is an opportunistic pathogen that sets upon the host when the normal immune defenses are disabled (Mulcahy et., 2014). It is a major problem in patients with cystic fibrosis, burn wounds, chronic wounds, chronic obstructive pulmonary disorder (COPD), nosocomial infections such as ventilator-associated pneumonia, catheter-associated urinary tract infections, and Asthma (Jurado-Martín et al., 2021). *Pseudomonas aeruginosa* PA1 (table 4.4.1), used in this study was found to be a multidrug resistant strain (Table 4.4.2). The findings of this study are similar to the findings of Li et al. (2016). *Pseudomonas aeruginosa PA1* was first isolated from a patient with respiratory tract infection in China (Lu et al., 2015). Multidrug resistance is positively correlated with the virulence of *P. aeruginosa* (Sonbol et al., 2015). Pyocyanin, a green metabolite of *P. aeruginosa* colonies in culture (Gellatly & Hancock, 2013) is one of the virulent factors that is produced by *Pseudomonas aeruginosa PA1* (Zhou et al., 2022). The hamsters in this study did not develop fever after inoculation with 0.2 ml of 1.5 x 10^8 cfu/ml of *Pseudomonas aeruginosa* PA1. Fever is defined as a temperature above 37.5^{o}C (Mackowiak et al., 202; Surana et al., 2022). According to the definition of fever above, no hamster had a temperature above 37.5 ° C (Table 4.4.4). Furthermore, Dunnett's t-test showed that there were no significant differences ($p > 0.05$) in the mean temperature for the hamsters

treated with distilled water, enalapril or losartan and then infected with *P. aeruginosa* compared control (hamsters treated with distilled water + PBS (table 4.4.5). A study by Johanson et al. (1985) found that an infectious dose of 5.0×10^6 cfu/ml of *Pseudomonas aeruginosa* was lethal to 65% of cystic fibrosis mice within 7.0 days, this lethal dose of *P. aeruginosa* is lower compared to the dose used in this study of 3.0×10^7 cfu/ml. In this study there was zero mortality after 14 days of observation of hamsters. *Pseudomonas aeruginosa* has an incubation period of 24 to 72 hours (Public Health Agency of Canada, 2011). The implication is that the hamsters were able to overcome the infection (Coalson et al., 1986) and that taking Enalapril or Losartan did not make the hamsters to develop fever after being infected with an opportunistic pathogen. A study by Kurahashi et al. (1999) concluded that injury to the alveolar epithelium is important in the release of pro-inflammatory mediators into the circulation that are primarily responsible for septic shock. In the case of this study, it is possible that there was no damage to the respiratory epithelium, therefore no pro-inflammatory mediators were able to flow into the circulatory system, this may explain why none of the hamsters presented with fever. Enalapril and Losartan did not cause the hamsters to develop fever. Thus, implying that enalapril and Losartan may not have a negative effect on the respiratory innate immune response to *Pseudomonas aeruginosa* infection at a dose of 3.0×10^7 cfu/ml.

CHAPTER SIX: CONCLUSION, RECOMMENDATIONS AND STUDY LIMITATIONS

6.1 Conclusion

The results of this study show that the administration of enalapril or losartan did not have a significant effect on the expression of FOXA2, MUC5AC and MUC5B. Furthermore, the administration of enalapril or losartan did not cause the hamsters to develop hyperplasia of the goblet cells or submucosal glands. Finally, the administration of enalapril or Losartan did not cause the hamsters to develop fever. The implication is that enalapril and Losartan did not have a negative effect on the tracheal mucosal barrier function to *Pseudomonas aeruginosa* infection at a dose of 3.0×10^7 cfu/ml. In general, the findings of this study show that administration of enalapril or losartan does not significantly increase the production of respiratory tract mucins. Therefore, the administration of these drugs will not reduce the innate immune response of the respiratory tract through the impairment of mucus production and thus will not increase the susceptibility of individuals to *Pseudomonas aeruginosa* infection.

6.2 Recommendations

The findings of this study show that patients suffering from respiratory infections and taking ACEIs, and ARBs should not stop taking these drugs if the major concern is the likelihood of impaired mucus production, as these drugs do not have a significant effect on the production of respiratory tract mucins and therefore are unlikely to negatively affect the respiratory innate immune response. More research is required to study the effect of marginal increases in MUC5AC and MUC5B on the viscoelasticity properties of respiratory mucus and mucociliary escalator.

6.3 Study limitations

1. Interpretation of the results is based on FOXA2, MUC5AC, and MUC5B, the effects of Enalapril and Losartan were not tested on other mucins.
2. Although ACEIs as a group have similar mechanisms of action, the interpretation of results may be limited to enalapril; similarly, although ARBs as a group have similar mechanisms of action, the interpretation of results may be limited to losartan.

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